IMMUNOLOGICAL AND ANTHELMINTIC STUDIES ON SETARIA CERVI INFECTION

ABSTRACT

THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
ZOOLOGY

By
ABDUL BAQUI
M. Sc., M. Phil. (Alig)

DEPARTMENT OF ZOOLOGY
ALIGARH MUSLIM UNIVERSITY,
ALIGARH
August, 1976

ABSTRACT

The filarioid worm, Setaria cervi was successfully implanted in laboratory white rats. Microfilariae appeared in peripheral blood circulation after a latent period of about one week and continued to persist for more than two months. Live worms survived in the peritoneal cavity for 4-6 weeks of the initial infection.

Both cellular and humoral changes were manifested by the infected rats. The highest microfilarial density was found to coincide generally with the greatest eosinophilic response. Serum protein analysis showed appreciable increase in α_1 - and γ - globulins which was non-specifically incriminated for the production of antibodies.

Known immunologic procedures such as complement fixation test, passive cutaneous anaphylaxis and precipitin tests were employed for the demonstration of complement fixing antibody, anaphylactic antibody and precipitins respectively in immune serum.

Cellular and humoral changes in rats following infection apparently seemed to confer immune status on rats which was characterised by the deleterious effects shown by the resistant animals upon the subsequent administration and residence of the parasite.

A number of anthelmintics namely diethylcarbamazine citrate (hetrazan), tetramisole, thiabendazole, tetrachloroethylene, piperazine citrate and acetylarsan were tested in rat-cervi system. Hetrazan and tetramisole proved quite effective and showed promising results. Both the drugs showed potent microfilaricidal property. Tetramisole was taken as the drug of choice in this filarial infection because of the fact it had shown its efficacy on adult worms also without any apparent side-effects. Other test chemicals were ineffective or too toxic to be used.

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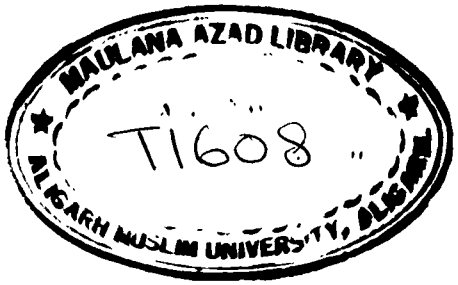
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Date August 11, 1976.

This is to certify that the thesis entitled "Immunological and anthelmintic studies on Setaria cervi infection" submitted by Mr. Abdul Baqui for the Ph.D. degree in Zoology has been completed under my supervision.

The work is original in nature and pursued by the candidate independently. I have permitted him to submit the thesis for the award of Ph.D. degree in Zoology.

Jamil A. Ansari.

(Jamil A. Ansari)

C O N T E N T S

ACKNOWLEDGEMENTS

I wish to express my deep sense of gratitude to Dr. Jamil A. Ansari for his invaluable guidance and selfless help without which this piece of work could not see the light of the day.

I am particularly indebted to Prof. S.H. Alam, Head, Dept. of Zoology for providing laboratory facilities and critical suggestions. Sincere thanks are also due to my colleagues Mondsur Khurshid A. Alavi and Mademoiselle Humaira Khatoon for their cooperation and help during the course of this work. Thanks are also due to Mr. Mohammad Wazir for his help in the collection of the parasite.

Last but not the least, I am grateful to the University Grants Commission for financial assistance.

INTRODUCTION

The filarial worm, *Setaria cervi*, is a cosmopolitan parasite inhabiting the peritoneal cavity of cattle. Incidence of this infection in our country is so high that approximately 70% of buffaloes and cows have been found to harbour this parasite. The parasite has been reported to cause tissue fibrosis, penetration and occlusion in the intestine of the natural host. The developing stages of the worm in strange circumstances may enter even the central nervous system of the natural host, and develop to partial maturity causing severe pathological condition known as 'Lumbar paralysis'. Accidental infection of this worm is also not uncommon among sheep and goats which may result into 'Cerebro-spinal nematodiasis'. Evidences are also available that many neurological syndromes of unknown aetiology in man and animals in tropics are due to this infection.

Studies on the parasite have been mainly related to its occurrence, host range, morphology and disease association. It is astonishing to note that no work has been done so far on the immunological and chemotherapeutic aspects of this parasite. In view of its heavy incidence, serious disease association and being zoonotic in nature, it has become the foremost

responsibility to find out measures which could restrict its activity and may be helpful in its eventual eradication. With these objectives present studies have been undertaken and efforts have been made to induce some sort of artificial immunity against this infection. Search for a suitable anti-filarial agent has equally been an important aim of the project. It is hoped that if both the steps were combined nicely, the term 'Setariasis' could be ruled out from the nature.

REVIEW OF LITERATURE

Host response to infections is manifold, and foremost expression is the change in the blood picture of the host. The change reflects the characteristic of a particular infection which is taken in conjunction with other findings in the diagnosis of a disease.

Van Someren (1938) described the blood changes in trichinosed rats, and noted marked degree of neutrophilic and eosinophilic responses. Holman and Pattison (1941) studied the blood picture of lambs suffering from parasitic gastritis, and reported clinical symptoms such as anaemia and eosinophilia. Similarly there are other reports also which indicate profound blood changes particularly in differential leucocytes following infections with Oesophagostomum, Bunostomum and Trichinella (Holler and Kissling, 1941; Delaume and Mayhew, 1941). Marill and Alcay (1945) carried out blood counts and bone marrow analysis of Senegalese infected with cutaneous onchocercinosis, and reported the red cell count perfectly normal while leucocytes showed considerable abnormal changes specially marked increase in eosinophils and a distinct decrease in neutrophils. Similarly, high degree of blood eosinophilia has been reported to be present in other filarial infections such as Wuchereria, Brugia (Buckley

1958; Buckley and Wharton, 1961; Joe, 1962). Djafar et al. (1960) reported significant eosinophilia in calves experimentally infected with Metyocaulus. Olson and Schultz (1963), Biocca (1969) reported high degree of eosinophilic response in guinea pigs, mice and monkeys experimentally infected with Toxocara canis, and which persisted for several months.

A number of reports has indicated that eosinophilia is fairly a diagnostic and clear-cut symptom in case of filarial infections. Whether any correlation between microfilaraemia and eosinophilia exists is not fully known. However, Harts and Van Dersar (1948) reported the tropical eosinophilia in filariasis associated with the microfilariae. Galliard (1954) reported that the periodicity of the microfilariae in the blood had no influence on variations of the eosinophil counts. Death and disintegration of adult worms might have led to tissue eosinophilia, sometimes resulting into eosinophilic abscess. Recently, Gidel and Brengues (1972) studied the reaction of Setaria labiatopapillosa infections both in normal and abnormal hosts. They observed a high increase of eosinophils in the normal host which was linked with the fluctuations in microfilarial density. Their experiments also showed that rats and guinea pigs developed eosinophilia following this infection, and which became more pronounced in sheep and goats and was entirely absent in rabbits.

Evidences are available which indicate that cellular factors play an important role in resistance of the host. Olsen (1959) reported marked increase in eosinophils in pleural cavity of cotton rats and white rats following Litomosoides carinii infection. Nagai and Subrahmanyam (1970) reported that cellular factors such as eosinophils, macrophages and lymphocytes played an important role in resistance (cell-mediated immunity) of white rats infected with Litomosoides rather than humoral antibodies. This was characterised by the local tissue response of immunologic nature around the microfilariae in pleural cavity.

Serum proteins are another important source of manifestation of an infection. Alterations in serum proteins present a diagnostic tool for various parasitic diseases. Change in certain fractions of globulin is used as an index of a specific parasitic infection. Wright and Oliver-Gonsales (1943) directly attributed the increase of γ-globulin fraction in rabbit due to Trichinella spiralis infection. Schwensen (1951) also observed an increase of γ-globulin fraction in human cases of trichinosis. Increase of β-globulin rather than γ-globulin has been reported by Leland et al. (1955) in rats infected with Nippostrongylus muris.

Zain-Eldin and Scott (1961) made comparison between resistant and susceptible hosts, to find factors responsible in conferring host resistance against a parasite. They observed

that α_1 -globulin in particular and γ -globulin were factors found elevated in white rats infected with Litomosoides carinii, and which protected them from infection; whereas other factors as α_2 -, β_1 - and β_2 -globulins were found to be significantly greater in gerbils, cotton rats and immature white rats than mature ones, but they did not give protection to them. Working with Capillaria obsignata infection in chicks Berghen (1966) reported an increase in total protein, total globulin and in all fractions of globulin particularly β - and γ-globulins, and a net decrease in A/G ratio. Recently, Singh et al. (1972) reported an increase in total globulin level and a decrease in albumin in buffaloes affected with microfilariasis.

All animals are more or less constantly exposed to a variety of infections ranging from microbes to helminth parasites. Fundamentally, the defensive mechanisms which are employed by the host against helminth parasites are essentially the same as those forces protecting animals against other infections. The same humoral and cellular defence systems are used by the host against all types of organisms.

Within the last thirty years much progress has been made in our understanding to immunity to nematodes. Ample evidences have now been accumulated to show that acquired immunity do occur in worm infestations in both natural and under experimental conditions. Some manifestation of acquired immunity to nematodes

has been demonstrated in Trichinella, Nippostrongylus, Ascaris, Litomosoides, Dirofilaria, Setaria, Haemonchus, Ewingia, Dictyocaulus. Most of the studies accumulated so far on acquired immunity against nematode infections have been confined to laboratory animals only. Very few reports are available where acquired immunity has been demonstrated in natural infections. Soulsby (1956, 1957) demonstrated immunity in calves against natural infections with Trichostrongylus and Dunostomum.

The common mode of immunizing procedure under laboratory conditions has been the administration of ova or immature stages of parasites into the host body through oral or subcutaneous routes (Fischthal, 1943; Soulsby, 1957; Taffs, 1964; Nakalin, 1973; Love et al., 1974). Some investigators have utilized adult worms in inducing immunization of animals. Ogilvie (1962) compared the resistance induced in rats against various developing stages of Nippostrongylus and adult worms, and reported that the adult worms were able to induce complete resistance. Similarly Okabe and Ono (1957), Kobayashi and Sumi (1973) immunised rabbits by intraperitoneal transplant of adult Setaria equina and Dirofilaria immitis. Scott and Macdonald (1951, 1958) were able to develop immunity in rats against Litomosoides carinii through mite induced infection or through inoculation of antigenic material, infective larvae or developing worms. Similarly, Olson et al. (1955), Ramakrishnan et al. (1962) were also able to induce immunity in rats against this worm. A new

approach of immunization against filarial worms was reported by Wong (1964) who was able to induce immunity artificially in dogs against Brugia pahangi and Microfilaria immitis by intravenous administration of live microfilariae suspended in blood-tyrode fluid- Freund's complete adjuvant mixture. Similar attempts have been made by Dhar et al. (1972)in albino rats with Litomosoides worms.

Several investigators have been successful in inducing immunity to nematode infections by the administration of extracts in the form of vaccine using adult worms or its larval stages as test material (Thorson, 1956; Soulsby, 1963; Jeska, 1967; Rubin et al., 1971; Wakelin and Selby, 1973). Sami (1970) demonstrated the production of reaginic antibodies in rabbits following sensitization with Microfilaria extract.

In the past majority of immunization and immunodiagnostic procedures have been directed towards the isolation of 'somatic antigens' and overlooked the best source of antigen viz., 'secretion and excretion' of living parasites. These metabolic products are known as 'excantigens'. It has been established that 'excantigens' obtained through pure in vitro cultures contain functionnal and potent antigens capable of evoking demonstrable immunity (Thorson, 1953; Campbell, 1955; Soulsby, 1957; Sadun and Norman, 1957; Mayhew et al., 1958; Alger, 1968).

Recently, the possibility of active immunization in animals against certain helminths has been demonstrated in laboratory as well as in field with irradiated parasitic stages (Jarret et al., 1958, 1960; Engelbrecht, 1961; Jarret and Sharp, 1963; Miller, 1964, 1966, Dee and Tripathi, 1968; Kadhim et al., 1973). Radiation vaccine has been used specially against sheep lungworm, Dictyocaulus filaria and Ancylostoma caninum, and found to be very effective. It has been reported that irradiation leaves the parasite pathologically less active and immunologically potent. Engelbrecht (1961) demonstrated the safety and potency of an irradiated vaccine against Dictyocaulus in calves.

Different immunization procedures as referred to above clearly indicates the fact that antibodies can be induced in animals by using one of these methods but when live worms are allowed to invade the animals, generally a good immunity is produced (Herlich et al., 1973). Dried and desiccated nematode materials have been reported to be often incapable of inducing protective or demonstrable immunity (Eisenbrandt and Ackert, 1940; Herlich et al., 1973). Such cases where worm extracts failed to induce immunity, Freund's complete or incomplete adjuvant have been extensively used along with the antigenic extracts in recent years (Silverman et al., 1962). The presence of Freund's adjuvant in antigenic mixture has been shown to elicit a proliferation of reticuloendothelial elements in mice

and guinea pigs (Laufer et al., 1959). Moriarty (1966) reported that the use of Freund's complete adjuvant developed the capability of producing antibodies and thus terminating the unresponsiveness or tolerant state of the host against a given parasite.

Nematode infections in general and filariases in particular have been recognised as a clinical entity for hundreds of years. It was only during the later part of the 19th century that the life cycle of the parasite, causes of the disease and pathogenecity etc. were studied. Diagnosis in filariases has been and is still mainly dependent upon the demonstration of microfilariae in blood or tissues, in spite of the fact that the parasitologic techniques are cumbersome, time consuming, often inadequate and unreliable. In order to overcome these problems various serologic techniques have been investigated, and were successfully employed in diagnosis of diseases, such as ascariasis, trichinosis, filariasis, and hookworm infection.

In most of the serologic techniques since antigen has been used hence its quality plays a major role in the specificity and sensitivity of an immunodiagnostic procedure. Various modifications have been introduced from time to time. In the beginning crude antigens prepared in saline were employed (Bozicevich and Hutter, 1944). Later, most of the investigators have used phosphate buffered saline antigen which was found to be most specific and sensitive (Bagni et al., 1968; Tanaka et al., 1968;

Ogunba, 1972). It has been reported that saline and buffered saline antigens appeared to be specific and contained all serologically active material. The non-specific factors present in the antigen have been mostly precluded by the delipidisation treatment with ether, and the antigen thus derived was most specific and sensitive (Chaffe et al., 1954; Manning and McFadzean, 1956a, 1956b). However, there are investigators who have found alcoholic extract most sensitive, reliable and superior antigen as compared to saline antigen. Such antigen has been in wide use in CFT and intradermal test (Lloyd and Chandra, 1933; Manning and McFadzean, 1956a, 1956b; Taffs, 1961). However, polysaccharide fraction has also been found to be reliable, specific antigen in certain serological test such as precipitin test (Okabe and Matsuse, 1957; Okabe et al., 1961). In some cases Kelcher's acid soluble fractions have been found to be effective and specific in test like haemagglutination and agar gel diffusion (Kagan and Bargai, 1956).

Sleeman and Muschel (1961) reported that lyophilized larvae of Trichinella extracted in sodium chloride and ethanol was found to be most specific and sensitive antigen in CFT.

Reliable immunodiagnostic procedures such as complement fixation test, indirect haemagglutination test, fluorescent antibody test, precipitin test, immunodiffusion, immunoelectrophoresis, passive cutaneous anaphylaxis, conglutinating complement absorption test, intradermal test are available for detection of a

variety of parasitic diseases. Each of the procedures has certain inherent advantages and limitations. Reports on few of the important tests are discussed here.

Passive cutaneous anaphylaxis (PCA) reaction has been used by investigators in basic studies on immune response to parasitic infections. The test is one of the most sensitive tools of all immunologic procedures, capable of detecting low levels of antibodies (Bagni et al., 1968). PCA procedure was for the first time introduced by Ovary and Bier (1955) which was further elaborated and modified by a number of investigators (Ogilvie, 1964; Ivey, 1967; Bagni et al., 1968; Kobayashi and Sarti, 1973) during the studies of dirofilariasis, Litomosoides and Trichinella infections. The basic and inherent advantage in the use of PCA procedure lies in the fact that it detects the development of homocytotropic or anaphylactic antibody which is characteristically produced in process in most of the helminthic infections, and having the capability of fixing to the tissues (Ogilvie, 1964). Fife (1971) reported that the PCA procedure is the most sensitive test of all serologic techniques and has important application in many unexplored areas. The author believes that this would be a most fruitful area for future research and would provide important knowledge concerning the immunopathology and host-parasite relationship in parasitic diseases.

The complement fixation test (CFT) has been widely used in the serodiagnosis of nematode infections in general and filarial infections in particular including both human and animal filariases. The CFT has been successfully employed in the serodiagnosis of wuchereriasis, loaiasis, dirofilariasis, ascariasis, Litomosoides carinii and several gastro-intestinal nematode infections (Culbertson et al., 1944; Ridley, 1956; Soulsby, 1956; Minning and McMahon, 1956; Rosseau-Baelde and Janssens, 1961; Taffs, 1964; Pacheco, 1966; Tanaka et al., 1969; Zahner, 1974). CFT has been reported to be specific and sensitive tool of detecting complement fixing antibody (Van Hoof, 1934; Huard and Tran Anh, 1950; Taffs, 1964). Fife (1971) reported that CF procedure is one of the more sensitive, versatile techniques in general use and is by far the most precisely standardized reproducible method available.

Precipitin test has been widely employed for a variety of immunological investigations. The test has been reported to be specific in a number of filarial infections (Okabe and Matsuse, 1956; Okabe et al., 1957, 1961). Culbertson et al. (1944a) reported that the test had a sensitivity equal to CFT and lower than the intradermal test. Okabe and Matsuse (1956) utilizing saline extracts of Setaria equina and Dirofilaria obtained excellent results with sera from patients and infected cattle. Kagan and Bargai (1956) used the precipitin test in diagnosis of trichinosis. They reported it to be suitable but less

sensitive than haemagglutination test used for antibody detection.

Immunodiffusion test has been reported to be highly sensitive and excellent tool for the diagnosis of many parasitic infections. The double diffusion in one or two dimension technique for the first time was developed by Oakley and Fulthorpe (1953), Ouchterlony (1958). The procedure permits direct comparison and identification of various antigens and antibodies in unknown mixtures which are allowed to diffuse in agar medium. Kagan and Bangai (1956) used the double diffusion in one dimension technique for the serodiagnosis of trichinosis. The technique has been reported to be very sensitive (Carpenter, 1965).

There are several ways of demonstration of immunity in animal host against a given parasite. One of the best methods of demonstration is the ability of the host to resist reinfection with the homologous parasite. Effects of immunity expressed by the host upon the parasite on subsequent infection, though not complete, have been reported to be death, inhibition of growth, and maturity, decrease in egg and juvenile production, elimination of worms and reduced pathological condition. Such direct evidence of immunity has been demonstrated in a number of nematode infections. Madun (1947), Dec and Srivastava (1961) have reported that chickens once exposed to Ascaridia galli eggs

became refractory to challenge reinfection after a month and were able to eliminate all worms of the test dose. Such immunity in chickens developed against the worms had been reported to last for 4 weeks to 12 months (Deo and Srivastava, 1961). In another study such a refractory state in kids and sheep has been reported to develop following primary infection with Haemonchus. During the challenge infection with the same parasite the animals showed considerable reduction in the faecal egg counts and worm burden as compared to control group (Stoll, 1929; Stewart, 1953, Soulsby, 1957). The elimination of worm burden, they reported, was due to immune response of the host called as 'self-cure' phenomenon. Rapport and Wells (1949) have demonstrated that as a result of immune effects Trichinella worms on reinfection in mice were found to be shorter, females containing fewer embryos and number of larvae present in the muscle were few. This could be attributed to the loss of fecundity in the females.

Evidences are also available which indicate that immune animals are comparatively safe, if not completely protected against secondary infection, and damage done is comparatively less severe. Taffs (1965) compared the damages done to the liver and lungs of guinea pigs and rabbits following Ascaris infection in resistant and non-resistant animals. He concluded that the damages caused by the parasite in the liver and lungs

in resistant animals were comparatively much less than that of non-resistant group. And greater degree of cellular response was observed in resistant group. Larvae in the liver were found to be trapped by inflammatory cells such as eosinophils, lymphocytes, neutrophils and macrophages. In another study Tewari et al. (1972) reported that strong resistance in animals could be conferred against Dictyocaulus by vaccination. The vaccinated animals, they reported, had significantly lower faecal larval count and better weight gains than control groups.

Evidences are also available which indicate that the primary exposure of animals to filarial worms render the hosts immune to challenge secondary infection. Scott and Macdonald (1951), Macdonald and Scott (1953) in a series of experiments showed that cotton rats acquired immunity against Litomosoides carinii following primary infection, and such immunity persisted for one or two years. The principal effects of immunity shown by these rats on secondary exposure of the parasites were (i) a reduction in number of worms due to failure of growth of the larval stages or to the death of developing worms (ii) a growth rate retardation. Wang (1964) reported that the dogs previously exposed to microfilariae of Dirofilaria induced the formation of antibodies which helped to destroy the microfilariae on subsequent administration. Similarly in another study Ah et al.

(1972) reported that the mongrel dogs previously exposed to Mirofilaria larvae became refractory to challenge infective larvae 60 days after previous immunization. This was manifested by the delay in appearance of circulating microfilariae in vaccinated group as compared to control group, and the number of microfilariae were 1/10th of those of control group.

Filariases have been considered for a long time as a great scourge to mankind and incurable diseases. Extensive investigations which followed later provided the possibility of chemotherapeutic control of the diseases. The chemicals commonly used as antifilarial agents are diethylcarbamazine citrate (DEC), tetramisole, levamisole, thiacetarsamide sodium, Hal. F, suramin sodium, neostibosan.

For the past several decades DEC has been used against a number of filarial infections and has shown a high rate of therapeutic and prophylactic values among most chemicals discovered so far. It is reported to be effective on microfilariae of Onchocerca, Litomosoides, Brugia and Setaria cervi (Burch and Ashburn, 1951; Hawking et al., 1950; Taylor and Terry, 1960; Zaman and Natarajan, 1973; Singhal et al., 1972). The drug has been used not only in laboratory trials and individual prophylaxis but also recommended in mass treatment of human filariases caused by Brugia and Wuchereria in endemic foci (Turner, 1959; Turner and Hodd, 1959; Sandosham, 1964).

The drug would have been a panacea in all filarial infections provided it did not have certain inherent disadvantages viz., (i) it is effective only against microfilariae and that too for a short period (ii) its side-reactions in many human infections. Cherry (1960) reported the drug to be highly efficacious in onchocerciasis, and recommended the use of prednisolone to control the side effects. Quite recently, Ban et al. (1975) recommended the use of DEC in filariasis, as being cheapest and best drug, in a slightly modified form. The authors reported that the common salt medicated with DEC minimised the side reactions caused by the drug and proved to be very rapid and efficient agent for the treatment of filariasis.

Some of the negative values of DEC has led to many investigators to find out a drug which could be effective on both adult worms and microfilariae. Suramin sodium has been found to be effective on the microfilariae and adult worms of Onchocerca volvulus (Bruch and Ashburn, 1951; Duke, 1968). But the drug showed toxicity and caused complications due to exfoliative dermatitis (Van Hoof et al., 1947). Several investigators have recommended the combined use of suramin with DEC to have synergistic action. Burch and Ashburn (1951) reported that the combined use of the two drugs brought immediate effect in onchocerciasis.

Another drug Mel. W was introduced by Friedheim and de Jough (1959). Dickerson and Thompson (1966) recommended the use

Hal. W for the mass treatment of filariasis. They reported the drug to be effective against adult Litomosoides worms with little action on microfilariae. The drug had a narrow therapeutic index against the worm in gerbils, and also produced certain side-effects. And there had also been several cases of arsenical encephalopathy following the use of Hal. W in treatment of onchocerciasis (Duke, 1966b).

Tetramisole and levamisole are new potent drugs being commonly used as a broad spectrum anthelmintics in a variety of nematode infections (Thienpont et al., 1966). More recently the drugs have been tested against filarial worms such as Brugia malayi (Zaman and Natarajan, 1973), Wuchereria bancrofti (Zaman and Lal, 1973), Brugia malayi (O'Holohon and Zaman, 1974), Litomosoides carinii (Laemler et al., 1971) and showed high degree of effectiveness. The drug proved quite promising in killing off the microfilariae as well as adult worms in laboratory trials on lorises and rats with Brugia and Litomosoides (Laemler et al., 1971; Zaman and Natarajan, 1973). Zaman and Natarajan (1973) compared the efficacy of levamisole with DEC in Brugia malayi infection and found the former being superior. It exhibited a high level of activity against microfilariae and adult worms. Toxic effects were minimum and obtained only at a very high dose. Zaman and Lal (1973) tested the efficacy of levamisole in Wuchereria

bancrofti infection and found that a dose of 120 mg was enough to cause a marked reduction in the number of microfilariae. In one patient some side effects similar to those with DEC developed at this dosage and was presumed to be due to the destruction of the parasite. Encouraging results have also been reported in Brugia malayi and Onchocerca volvulus by the use of this drug (O'Nolohon and Zaman, 1974).

Culbertson et al. (1946) reported the therapeutic efficacy of neostibosan in Wuchereria bancrofti adult worms with no toxic effects to the patients except symptoms like nausea and vomitting.

Thiacetarsamide sodium has been reported to be an effective drug in treatment of canine heartworm disease. The drug was first introduced by Otto and Maran (1947). The effectiveness of the drug as a filaricid has been clearly demonstrated in Microfilaria immitis infection being effective on both adult worm and microfilariae (Jackson, 1963).

Lammler et al. (1971) made a comparative screening of a number of antifilarial agents namely DEC, tetramisole, levamisole, cyclohexane carboxylic-acid-N-methyl piperazine citrate, tetrahydroxy renecarboxylic acid-N-methyl piperazine citrate, metrifonate against Litomosoides carinii infection in Mastomys natalensis. The cycloaliphatic carboxylic-N-methyl piperazine

derivatives showed microfilaricidal effects with no activity against mature parasite. And the microfilaricidal effects were similar to those caused by DEC. Metrifonate (negrvon) was also found to be effective on microfilariae only. Broad spectrum anthelmintics tetramisole and levamisole were highly effective on microfilariae as well as adult worms.

Many other drugs have also been used in filarial infections including thiabendazole, bephenium hydroxynaphthoate, tetrachloroethylene, piperazine citrate. But they were either ineffective or too toxic (Unochiri, 1966; Lemmler et al., 1971; Singhal et al., 1972). Singhal et al. (1972) reported that 3-acetamido-4-hydroxy phenyl arsonic acid was also an effective drug in rat-cervi system with no apparent side effects.

MATERIALS AND METHODS

Parasite and experimental host:

Adult worms of Setaria cervi, were obtained from the peritoneal cavity of freshly slaughtered water buffaloes, Bos bubalis. The worms collected in physiological saline, were washed several times to remove extraneous matter. Prior to the transplant these were transferred to an incubator maintained at 37°C.

White rats, were used as the test animal. They were bred in the laboratory, and every care was taken to prevent all possible external infections. Rats utilised in the experiments were almost of the same age group and weight.

Establishment of infection:

Implant of the worms was made via laparotomy. A fine incision was made in the body wall of anaesthetised rats and worms were slipped into the peritoneal cavity. At the close of the experiment the opening was sutured and iodoform-merbromin solution was applied locally. The rats inoculated in this way continued to be healthy, and no internal treatment was given to them.

In order to assess the establishment of infection and level of microfilaraemia blood samples of infected rats were taken regularly at 6 - 8 P.M. The quantity of blood, always maintained, has been 1 mm^3. The blood, readily obtained by severing the tip of the tail, was expelled over the slide and erythrocytes were lysed by adding 2-3 drops of distilled water. Microfilarial count was done under the microscope. Microfilaria positive blood was used in all the experiments.

Optimal immunizing dose:

In order to find out the optimal dose of the parasite, various test doses of worms ranging from 2-12 of both sexes were implanted in a number of rats. It was observed that worm burden of more than 5 led to early appearance of microfilariae and prolonged patent period. But they showed great disadvantage and limitations as the mortality rate among rats was increased considerably. An optimal dose was taken as that one which gave long duration of microfilaraemia, short latent period and low mortality among rats. Three female and two male worms were found to be ideal combination and was adopted as an optimal dose in various experiments.

Part A

BLOOD ALTERATIONS

I. CELLULAR RESPONSE

INTRODUCTION

Response to infections is manifested by a number of ways. Foremost expression is the change in the formed elements of the blood and this sometimes reflects a specific characteristic of a particular infection which is taken in conjunction with other findings in diagnosis of a disease. Extensive studies have been made on this aspect but mostly related to host infections with microbes and protozoan parasites. Comparatively, little experimental work has been done on nematode infections. Various investigators have reported varied leucocytic response in nematode infections.

The present study on *Setaria servi* infection in white rats has been under taken in order to ascertain leucocytic changes which may provide a valuable information.

MATERIALS AND METHODS

Differential blood count was carried out in 30 infected rats. Regular count was made every 7th day on all rats for a period of over two months. Blood was readily obtained by

severing the tip of the tail. Staining of the blood smear was made with Giemsa stain. A simple differential count of neutrophils, lymphocytes, eosinophils, monocytes and basophils was made. Total leucocyte count was not carried out hence the study only deals with the relative variations in leucocyte ratios. A simple differential count (DLC) was also carried out in a number of normal uninfected rats served as control, and an average of the data was taken.

In order to assess the level of microfilaraemia during the course of infection, blood samples of the infected rats were also taken at weekly intervals for the period until disappearance of microfilariae. The quantity of blood, always maintained, has been 1 mm^3. The blood was expelled over the slide and the erythrocytes were lysed by adding 2-3 drops of distilled water. Microfilarial count was made under the microscope.

RESULTS

Microfilariae appeared in the peripheral blood circulation of all rats with a latent period of 7±2 days. The microfilarial count showed a consistency of 1-3 in early period. From 2nd week microfilarial population started increasing. In the 3rd and 4th weeks, microfilariae were found to be maximum in blood circulation, thereafter it started declining with slight fluctuations

from high to low. Such condition remained constant in almost all rats with slight variations. Microfilariae persisted in blood circulation ranging 9-10 weeks. The maximum microfilarial range was found to be 17-26 and an average figure of 21/mm^3 of blood (Table 1).

The differential leucocyte counts on control rats gave, on an average, the following figures (a typical normal counts).

Lymphocytes	48-70%	Average	59.0%
Neutrophils	28-41%	"	34.5%
Eosinophils	2- 4%	"	3.0%
Monocytes	1- 5%	"	3.0%
Basophils	0- 1%	"	0.5%

The blood counts of the rats infected with Setaria cervi showed that these sharply reacted with this filarial worm. Results obtained are given in Table 1, and the average figures of leucocyte ratios are presented together with microfilaraemia. No change was noticed in the Ist week of initial infection except in two rats in which aneosinophilia was observed in the peripheral blood circulation. From 2nd week neutrophils started increasing and reached its maximum by 3rd and 4th weeks, and thereafter declined to normal range with slight fluctuations. By the 3rd week eosinophils started increasing and reached its maximum by 4th week and then declined. But even after its decline, high degree of eosinophilia (9.2 - 10.6) was maintained

for two more weeks. First 5-7 weeks lymphocytes showed a gradual increase after the decline and reached its maximum by 7th week, and then came to normal limit range. Eosinophilic maxima varied with the individual animal. In most of the rats the eosinophilic maxima were obtained during the period (4th week) in which maximum microfilarial concentration was noted in the blood. But in few cases maximum eosinophilic response was observed during 5th and 6th weeks. By the end of 10th week, all the three types of leucocytes mentioned came to normal limits (Table 1: Figure 1).

Monocytes and basophils throughout the infection in all rats did not show any appreciable change. All the rats examined for a period of over two months showed more or less same pattern of response in the blood. In all cases leucocytic response with regards to neutrophils, eosinophils and lymphocytes has been more significant. Neutrophils rose to 48-60% and lymphocytes fell to 45-33%. Similarly lymphocytes increased to 68-80% and neutrophils came to 22-10%. Thus there was a reversal mechanism persisting in the proportion of neutrophils and lymphocytes which became more marked with the beginning of 3rd week and continued till 7th week from the initial infection (Fig. 1).

Table 1. Differential leucocyte count and microfilaraemia of infected rats.

Weeks after infection	Neutrophils		Lymphocytes		Eosinophils		Microfilariae /mm^3 of blood	
	% Range	Average	% Range	Average	% Range	Average	Range	Average
1	30 - 40	34.6	55 - 66	61.4	0 - 4	2.6	1 - 3	2.4
2	33 - 45	39.6	50 - 64	53.8	2 - 6	4.2	4 -10	6.8
3	48 - 60	52.4	33 - 45	39.2	5 - 9	6.8	15 -20	18.8
4	39 - 50	42.4	40 - 50	41.8	8 -16	14.2	17 -26	21.0
5	29 - 38	34.6	51 - 61	54.2	7.-12	9.2	7 -13	10.6
6	17 - 27	21.0	56 - 70	64.2	8 -13	10.6	4 -10	5.0
7	10 - 22	16.2	68 - 80	75.6	6 - 8	6.8	4 - 7	5.5
8	25 - 36	31.6	55 - 67	61.4	5 - 8	5.8	3 - 5	3.5
9	33 - 40	35.8	50 - 60	54.2	3 - 7	4.8	0 - 3	0.6
10	29 - 34	33.4	55 - 61	59.0	2 - 5	3.4	0 - 1	0.2

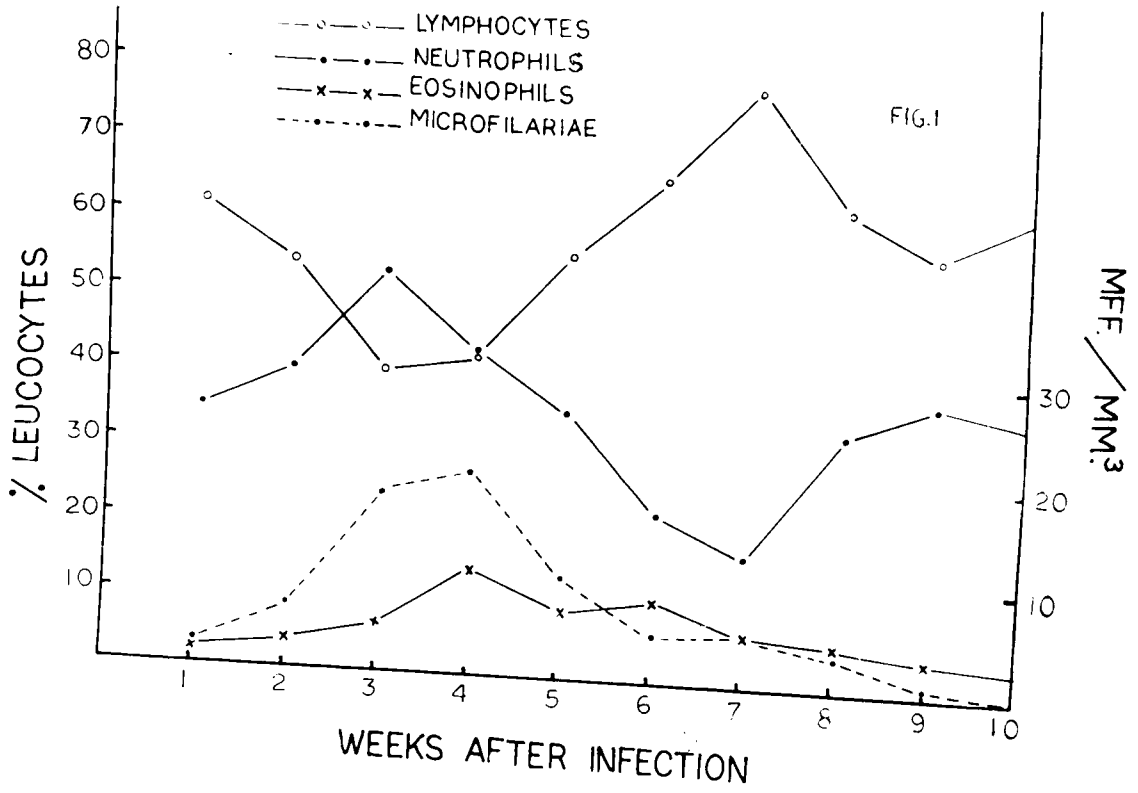


Fig. 1. Leucocyte ratios and microfilaraemia in the blood of infected rats.

DISCUSSION

Although differential leucocyte count (DLC) of normal rats varied slightly from day to day in the same animal, the average figures were found to be in accordance with those earlier worker like Van Somaren (1938).

DLC of infected rats indicated positive changes. In the initial stage the change was insignificant except in two rats in which eosinophils were entirely absent. Such a period of aneosinophilia appears to be a non-specific response.

In almost all cases the microfilariae appeared in blood circulation after a latent period of one week. A gradual rise in neutrophils from 2nd week till 4th week explains that these being phagocytic in nature and first mobilised cells, they come into action in greater number to repel the invading microfilariae during early phase of infection. A similar observation has also been reported in case of Toxocara canis infection in mice and monkeys (Masseru, 1969).

Eosinophils increased by the 3rd week and reached its maximum by 4th to 6th weeks. An increase in eosinophils has also been reported in man suffering from onchocerciasis and Brugia malayi infection (Harrill and Alony, 1945; Buckley and Wharton, 1961). It has been found that even after its decline

after 6th week eosinophils never reached to normal limit, but a constant low grade eosinophilia persisted for two more weeks. The persistence of eosinophilia at lower level for certain periods might be related to the release of substances from dead worms, larvae and their metabolites in the host. Van Somoren (1958), Miseeru (1969) reported chronic eosinophilia in rats, mice and monkeys following Trichinella spiralis and Toxocara canis infections, and which persisted for a long time even after its decline from the maximum peak. Olson and Schultz (1963) attributed the rise and fall of eosinophils in guinea pigs following Toxocara canis infection to the qualitative or quantitative change in worm antigen. The inability of the host to acquire resistance to nematode infections has been correlated with a relatively poor eosinophilic response (Jafar et al., 1960). They reported significant eosinophilia in calves surviving Dictyocaulus infection in contrast to the low eosinophilic response of these animals with severe clinical symptoms. Similar inference has been made by Baker (1962) in mice infected with Hematospiroides dubius.

Lymphocytes showed a slight increase from 5th to 7th weeks and then came to normal limit range. Miseeru (1969) has concluded that in mice and primate, lymphocytes (and eosinophils) take part in the production of antibodies and aid in the development of resistance to invading Toxocara canis larvae. Correlating these facts it appeared that lymphocytes (and eosinophils)

might be aiding in removal of microfilariae from the blood by taking part in the host's defence mechanism. Monocytes and basophils showed no appreciable change in all cases.

Hartz and Van Dersar (1948), Joe (1962) tried to establish a relationship between the appearance of tropical eosinophilia and microfilaraemia, and reported the tropical eosinophilia associated with microfilariae. Galliard (1954) reported that the periodicity of the microfilariae in the blood had no influence on the variations of eosinophil counts. Death and disintegration of adult worms might have led to tissue eosinophilia, sometimes resulting into the eosinophilic abscess. Buckley and Wharton (1961) reported that even in the presence of high degree of circulating eosinophils in man exposed to Brugia malayi, microfilariae were not present in the blood. The authors attributed it as an unexplained fact. Wong (1964) while working on the microfilaraemia in dogs reported that a high titer of antibodies in the host was probably responsible for the absence of microfilaraemia in some filarial diseases.

Very recently, Gidel and Dreyfuss (1972) working on Setaria labiatopapillosa infection in normal and abnormal hosts, observed a high increase of eosinophils in the natural host, and linked the fluctuations in the microfilarial density with the changes in the eosinophil counts. They also observed

eosinophilia in abnormal hosts. In the present study also, the highest microfilarial density in the blood of infected rats generally coincided with the period of greatest eosinophilic response, which is a clear indication that a correlation does exist between the appearance of microfilariae in blood and circulating eosinophils.

II. SERUM PROTEIN CHANGES

INTRODUCTION

Serum proteins are important source of manifestation of an infection. Alterations in serum proteins present a diagnostic tool for various parasitic diseases. Changes in serum proteins particularly in certain fractions of globulin have been used as an index of a specific immunological response. Wright and Oliver-Gonzales (1943), Schwenzen (1951) directly attributed the increase of γ -globulin in rabbits and man following Trichinella spiralis infection to antibody production. Increase of β -globulin rather than γ -globulin has been reported by Leland et al. (1955) in rats infected with Nippostrongylus muris. Sein-Eldin and Scott (1961) reported that α_1 -globulin and γ-globulin were the factors found elevated in white rats infected with Litomosoides carinii, and which protected them from infection. Several investigators have attributed the hyperglobulinemia accompanied by hypoalbuminemia condition in parasitic infections as a result of liver involvement or intestinal damage (Gutman, 1948; Berghen, 1966; Singh et al., 1972).

The above resume reveals a fact that no specific study regarding the serum protein changes following Hateria servi

infection has been done. With this view the present work has been undertaken to find out changes in serum proteins following induced Setaria cervi infection.

MATERIALS AND METHODS

Serum protein changes were studied in eighty infected rats and ten control rats. Five worms were implanted surgically into the peritoneal cavity of white rats. In control rats no worms were implanted, and only a fine incision was made in the body wall and stitched. Infected rats were divided into eight batches. The study continued for two months. The rats were sacrificed at weekly intervals, and blood was collected aseptically by cardiac puncture. Blood thus collected was allowed to clot at room temperature for an hour and then transferred to a refrigerator. The serum was finally separated by centrifuging the blood at 3000 rpm for 10 minutes. Serum protein analysis was carried out immediately.

Quantitative analysis of total serum protein and serum albumin was carried out by the known 'Biuret test' described by Wootton (1964). Serum globulin was determined by subtracting the values of albumin from total protein. For qualitative analysis, agar-gel electrophoresis technique based on the method described by Bergsten (1966) with little modifications was used.

All sera were analysed on Systronics horizontal electrophoresis apparatus. A barbitone buffer (pH = 8.6) was used. 1% hot Difco agar solution, dissolved in barbitone buffer (pH = 8.6), was layered over microscope slides. When the agar was hard, a small piece of Whatman filter paper No. 1 (size 0.1 x 1 cm) was soaked in the 0.02 ml serum, and applied on the slide towards the negative end at the junction of 1/3 and 2/3. The slides were then subjected to 200 volts (20-30 ma) for 2½ hours in electrophoresis apparatus. After running, the slides were taken out from the apparatus, and fixed in pure methanol for 10 - 15 minutes. Later, these were stained in 0.5% Amidoschwarz 10B solution for 15 minutes. After staining, the slides were washed with three changes of methanol-acetic acid-water solution during a period of 10 - 15 minutes in each wash. Washing solutions were prepared in three different concentrations. The slides were then put upside down on filter paper soaked in distilled water to remove the excess of stain, and to distinguish the various bands.

After drying, the slides were placed in Systronics densitometer for qualitative estimation of different fractions of serum protein. It was carried out by taking optical density readings from densitometer at every one millimeter distance of the slide. The optical density readings were plotted on a graph

paper against the distance in millimeter. The area covered by each fraction of serum protein was separated by drawing a perpendicular line from the valley to the base line, and then, percentage area covered by each fraction was worked out.

Quantitative analysis for total serum protein and albumin was carried out by using 'Biuret method' which proved to be easy and ideal for such estimation. Three test tubes marked T (Test), B (Blank) and S (Standard) were taken. 0.2 ml of serum was aspirated in test tube T and made upto 3 ml by adding 2.8 ml distilled water. In test tubes B and S 3 ml distilled water and 3 ml standard protein solution (0.5 gm/ 100 ml bovine albumin) were taken respectively. 5 ml Biuret reagent was added to each tube, mixed well and kept in a water bath maintained at 37°C for 10 minutes. After cooling, compared the colours at 540 mμ. Total serum protein was calculated as follows:

$$\text{Total serum protein} = \frac{T - B}{S - B} \times 0.5 \times 15 \text{ gm/100 ml}$$

The total albumin in serum was carried out by adding 0.5 ml serum to 5.5 ml of 26% sodium sulphate solution in a 10 ml centrifuge tube and mixed by inversion. Added 1 ml of ether-span reagent, stoppered the tube and centrifuged for 10 minutes. It caused globulin precipitate to separate as a

disc at the ether-water interface, while the use of 10 ml tube restricted the air space and reduced the denaturation at the air-liquid interface. The globulin disc was gently pushed down with the tip of a pipette and withdrew 3 ml of the sub-natant layer. The concentration of albumin was 1/12 of that in the original serum. 0.5 ml of Biuret reagent was added, mixed well and warmed in a 37°C water bath for 10 minutes. After cooling compared the colours at 540 mμ. In serum albumin test, like total protein test, 3 ml of standard protein solution and 3 ml of distilled water (Blank) were taken. Serum albumin was calculated as follows:

$$\text{Serum albumin} = \frac{T - B}{S - B} \times 0.5 \times 12 \text{ gm/100 ml}$$

Analysis of the electrophoresis data converted to concentration of the protein components in terms gm/100 ml from the quantitative Biuret test, gave a better evaluation of the the actual changes which have taken place following infection.

In order to assess the level of microfilaraemia and condition of adult worms during the course of infection, rats were autopsied at weekly intervals. Blood samples taken for microfilarial count were always 1 mm^3.

RESULTS

Microfilariae appeared in the peripheral blood circulation of all rats with a latent period of 7 ± 2 days. The microfilarial count showed a consistency of 1-4 in early period of infection. From 2nd week microfilarial population started increasing and reached its peak during 3rd and 4th weeks, thereafter it started declining with slight fluctuations from high to low. The population density of microfilariae during the course of infection is presented in Table II.

Averages of the relative values of serum proteins and its fractions of control and infected rats are presented in Table I. A typical pattern of serum proteins of control and infected rats are presented by Figures 1 to 9.

Variations in the 1st week in infected rats were not very substantial, and the amount of total protein, albumin and total globulin remained almost unchanged. Globulin fractions showed slight decrease with the exception of β-globulin which increased to 20.9%. Variations in the 2nd week were slightly significant and showed changes in the amount of total protein and protein fractions. Albumin decreased to 7.0% and there was a positive increase in all globulin fractions particularly in γ-globulin which showed an increase of 48.1%. Data of the

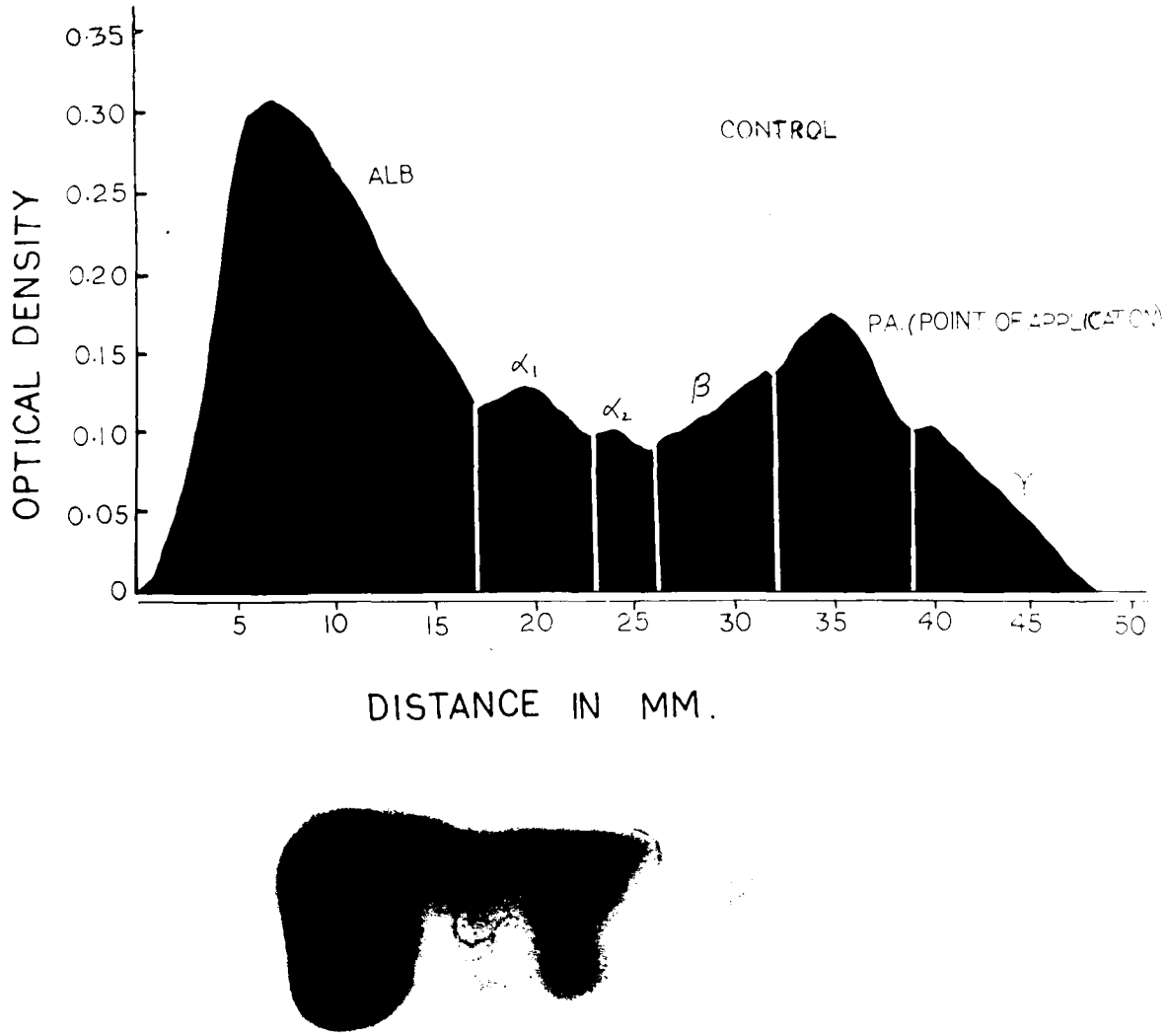


Fig. 1. Electrophoretic pattern of the control rat serum.

Table I. Electrophoretic analysis of rat serum (gm/100 ml).

Infected rats (Batches)	Weeks after infection	Total protein (± SE) % change	Albumin (± SE) % change	Total globulin (± SE) % change	GLOBULIN α_1 (± SE) % change	GLOBULIN α_2 (± SE) % change	GLOBULIN β (± SE) % chan
Control	-	5.80 ± 0.09	3.42 ± 0.03	2.38 ± 0.04	0.66 ± 0.02	0.32 ± 0.03	0.86 ±
1	1	5.65 ± 0.09 -2.5	3.29 ± 0.04 -3.8	2.36 ± 0.03 Insignificant	0.54 ± 0.01 -18.1	0.24 ± 0.01 -25.0	1.04 ± +20.9
2	2	6.18 ± 0.08 +6.5	3.18 ± 0.08 -7.0	3.00 ± 0.06 +26.0	0.80 ± 0.07 +21.2	0.40 ± 0.02 +25.0	1.00 ± +16.2
3	3	6.26 ± 0.09 +7.9	2.96 ± 0.03 -13.4	3.30 ± 0.03 +38.6	0.90 ± 0.07 +36.3	0.42 ± 0.03 +25.0	1.10 ± +27.9
4	4	6.96 ± 0.08 +20.0	2.82 ± 0.04 -17.5	4.14 ± 0.09 +73.8	1.32 ± 0.02 +100.0	0.47 ± 0.03 +46.8	1.10 ± +27.9
5	5	6.40 ± 0.09 +10.3	2.55 ± 0.07 -25.4	3.85 ± 0.05 +61.7	1.29 ± 0.01 +95.4	0.55 ± 0.01 +71.8	0.95 ± +10.4
6	6	6.24 ± 0.06 +7.5	2.80 ± 0.01 -18.4	3.44 ± 0.04 +44.5	1.00 ± 0.01 +51.5	0.54 ± 0.03 +68.7	1.00 ± +16.2
7	7	6.00 ± 0.06 +3.4	3.14 ± 0.03 -8.1	2.86 ± 0.02 +20.1	0.77 ± 0.04 +16.6	0.42 ± 0.02 +31.2	0.90 ± +4.6
8	8	6.00 ± 0.07 +3.4	3.30 ± 0.04 -3.5	2.70 ± 0.05 +13.4	0.77 ± 0.01 +16.6	0.31 ± 0.02 Insignificant	0.93 ± +8.1

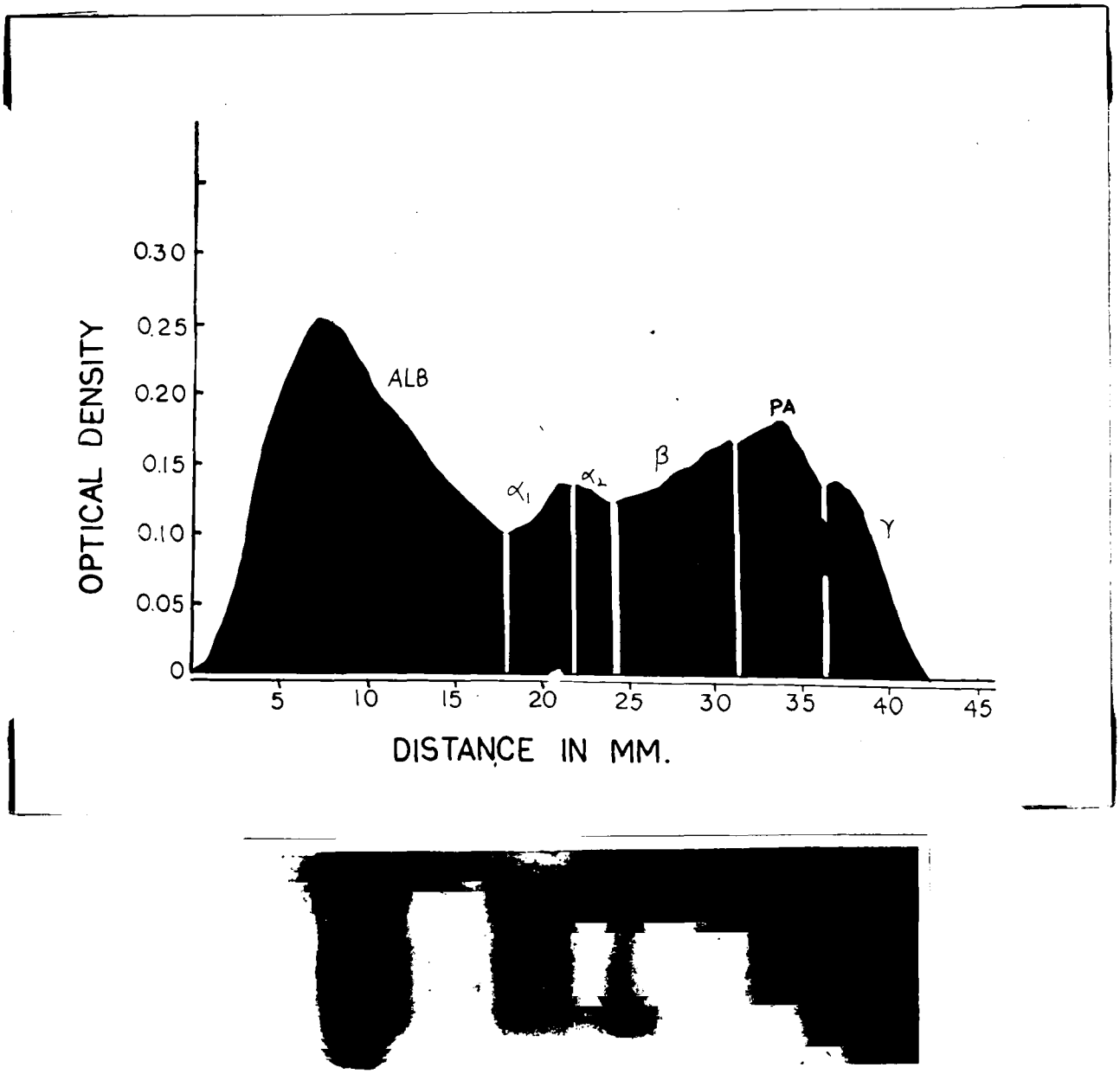
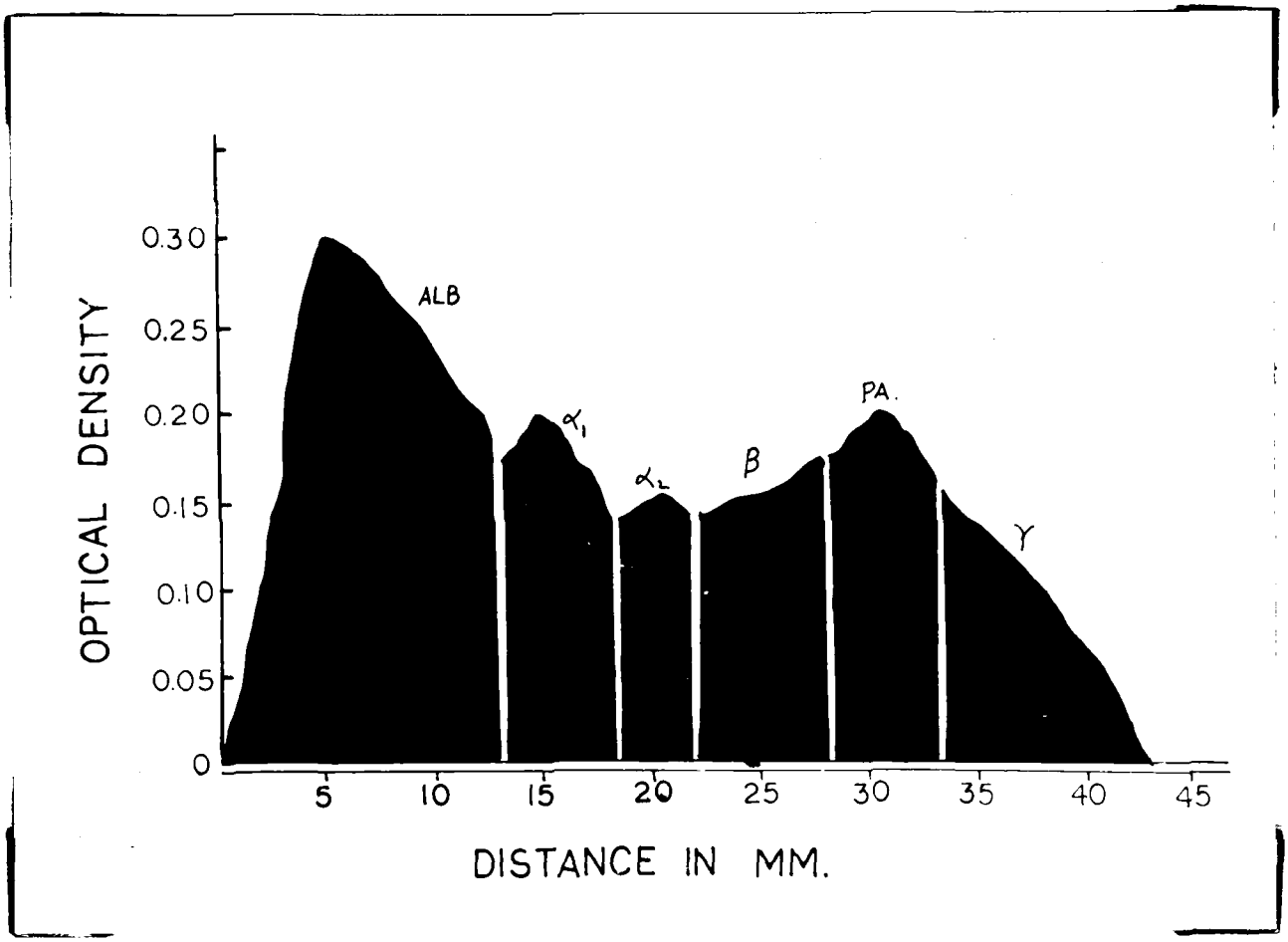


Fig. 2. Electrophoretic pattern of the infected rat serum (Ist week).

Table II. Population density of microfilariae in blood.

Recipient rats (Inbred)	Mean microfilarial density							
	Duration of microfilaraemia in weeks							
	1st	2nd	3rd	4th	5th	6th	7th	8th
1	3.1	-						
2	1.5	5.0	-					
3	4.0	4.3	9.2	-				
4	1.9	7.1	5.7	13.0	-			
5	2.3	6.5	13.0	21.0	11.0	-		
6	3.0	9.3	13.6	17.3	11.3	6.7	-	
7	2.2	5.0	9.9	15.4	13.3	6.5	3.2	-
8	3.1	4.3	7.9	17.7	6.9	9.7	3.4	3.0



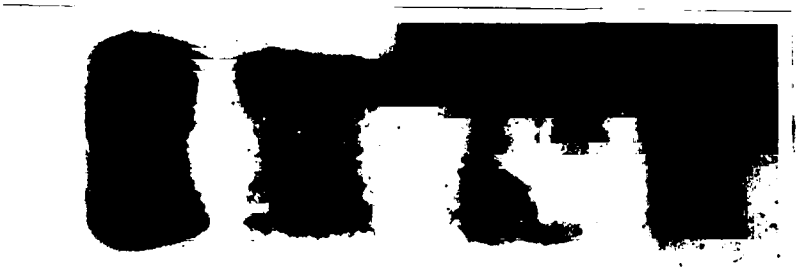


Fig. 3. Electrophoretic pattern of the infected rat serum (2nd week).

3rd week showed marked elevation in γ -globulin (66.6%), whereas α_1 - and β -globulins showed an increase of 36.3% and 27.9% respectively. During 4th week marked elevation in all globulin fractions particularly α_1 - and γ -globulins were noted which increased to 100.0% and 131.4% respectively. In 5th week all conditions of 4th week persisted with slight fluctuations. During 6th week α_2 -globulin only showed a high level whereas other globulin fractions showed a comparative decrease, and during 7th week all globulin fractions showed a further decrease. By 8th week total protein, albumin, total globulin and its fractions showed a trend towards normal levels. α_2- and β -globulins showed almost normal values while α_1 - and γ -globulins showed only an increase of 16.6% and 27.7% respectively.

The amount of total protein throughout the infection indicated progressive increase except in Ist week where it decreased to 2.5% which was almost negligible. The highest increase in the amount of total protein was noted in 4th week (20.0%), thereafter it indicated relative decrease, and by the end of 8th week it almost touched the normal level (only an increase of 3.4%).

Albumin throughout the infection showed almost a progressive decrease, and by the 5th week it decreased to 25.4%. The highest level of total globulin was noticed from 3rd to 6th

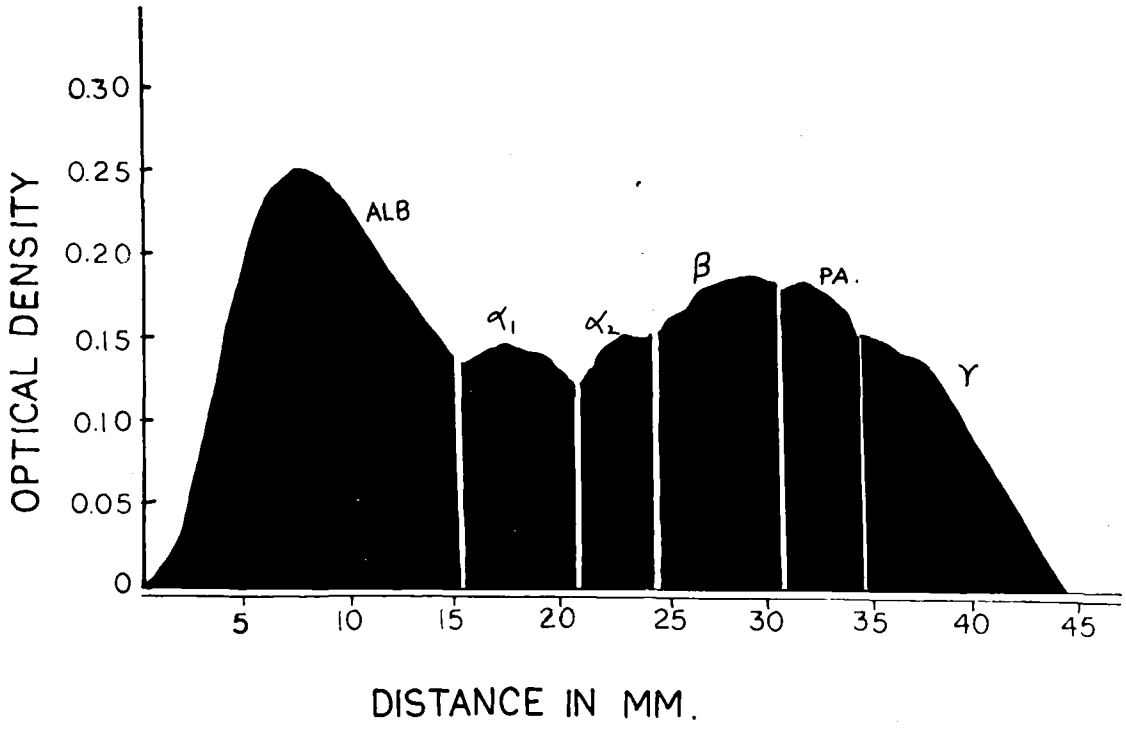


Fig. 4. Electrophoretic pattern of the infected rat serum (3rd week).

weeks. The albumin and globulin ratio (A/G ratio) of control rats with infected groups was comparable. Throughout the infection the A/G ratio was progressively diminished excepting last three weeks of infection. In the Ist week the decrease was almost negligible (2.7%). Highest decrease in A/G ratio was found during 4th and 5th weeks.

Autopsy carried out at weekly intervals showed that the adult worms survived in the peritoneal cavity of the rats for about 4-6 weeks, and thereafter they started disintegrating. Dead and disintegrating worms were found to be encapsulated in the peritoneal wall, intestinal mesentries and in the genital region.

DISCUSSION

Various reports indicate that a rise in γ -globulin is a significant reflection of humoral response to an infection: and that globulins in general and γ -globulin in particular have some bearing on the production of antibodies. In view of this fact, elevation in different fractions of globulin particularly α_1 - and γ -globulins in rat-cervi system, may also be incriminated for the production of antibodies. Wrights and Oliver-Gonzalez (1943) attributed the rise in γ -globulin

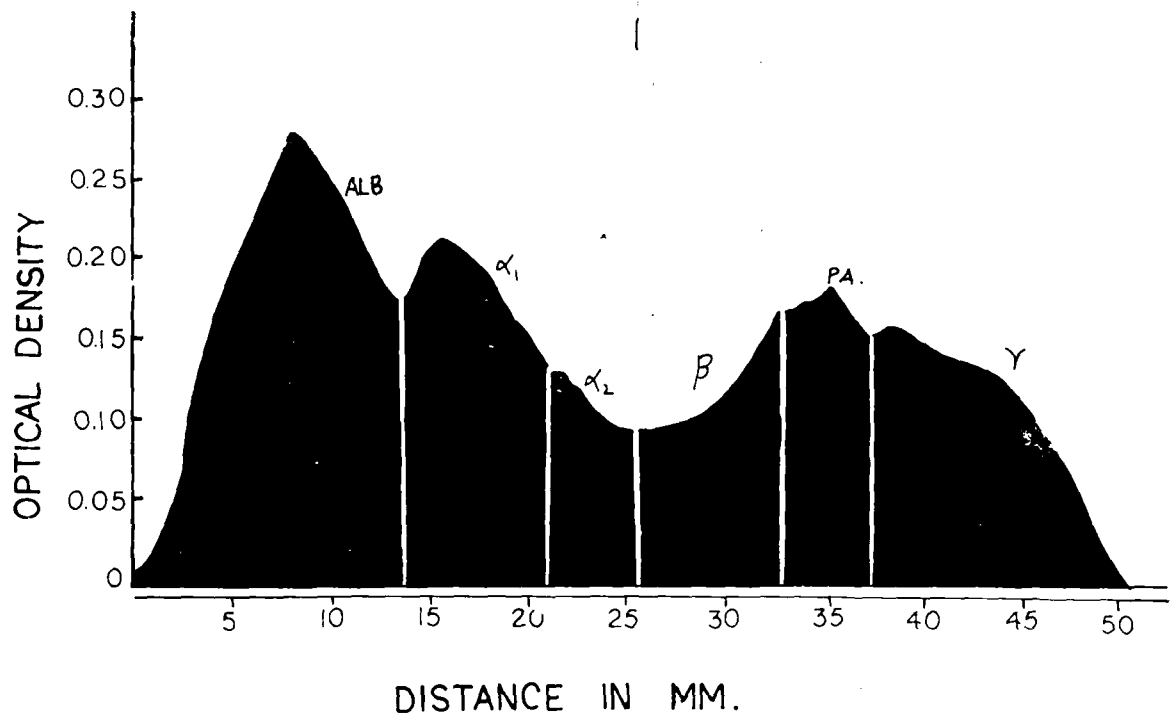


Fig. 5. Electrophoretic pattern of the infected rat serum (4th week).

during the course of Trichinella spiralis infection as a direct result of antibody production and reported that as the immunity developed there was marked increase in the amount of γ -globulin in the period in which larval immunity reached its peak. According to them this γ -globulin particularly contained antibodies effective against larvae and adult worms. Schwonzen (1951) also observed similar results in human cases of trichinosis. Zein-Eldin and Scott (1961) working on Litomosoides carinii infection in rats indicated that α_1 - and γ -globulins were the two important factors which were involved in resistance. The change in β - and γ -globulins was taken by Berghen (1966) in Capillaria obsignata infection in chicks as an index of specific immunological response, and indicated manifestally the existence of a supply of specific antibodies.

In view of these facts the obvious rise in globulin fractions from 2nd upto 6th weeks may be a reflection of immunity against microfilariae by virtue of which they started diminishing just after 4th week. The action of antibodies on the microfilariae has been reported by Won ; (1964). The author suggested that a high titer of antibodies is responsible for the absence of microfilaraemia in some filarial diseases. Maximum antibody concentration in experimental infection of rabbits with Setaria equina has been recorded by Okabe and Ono (1957) from

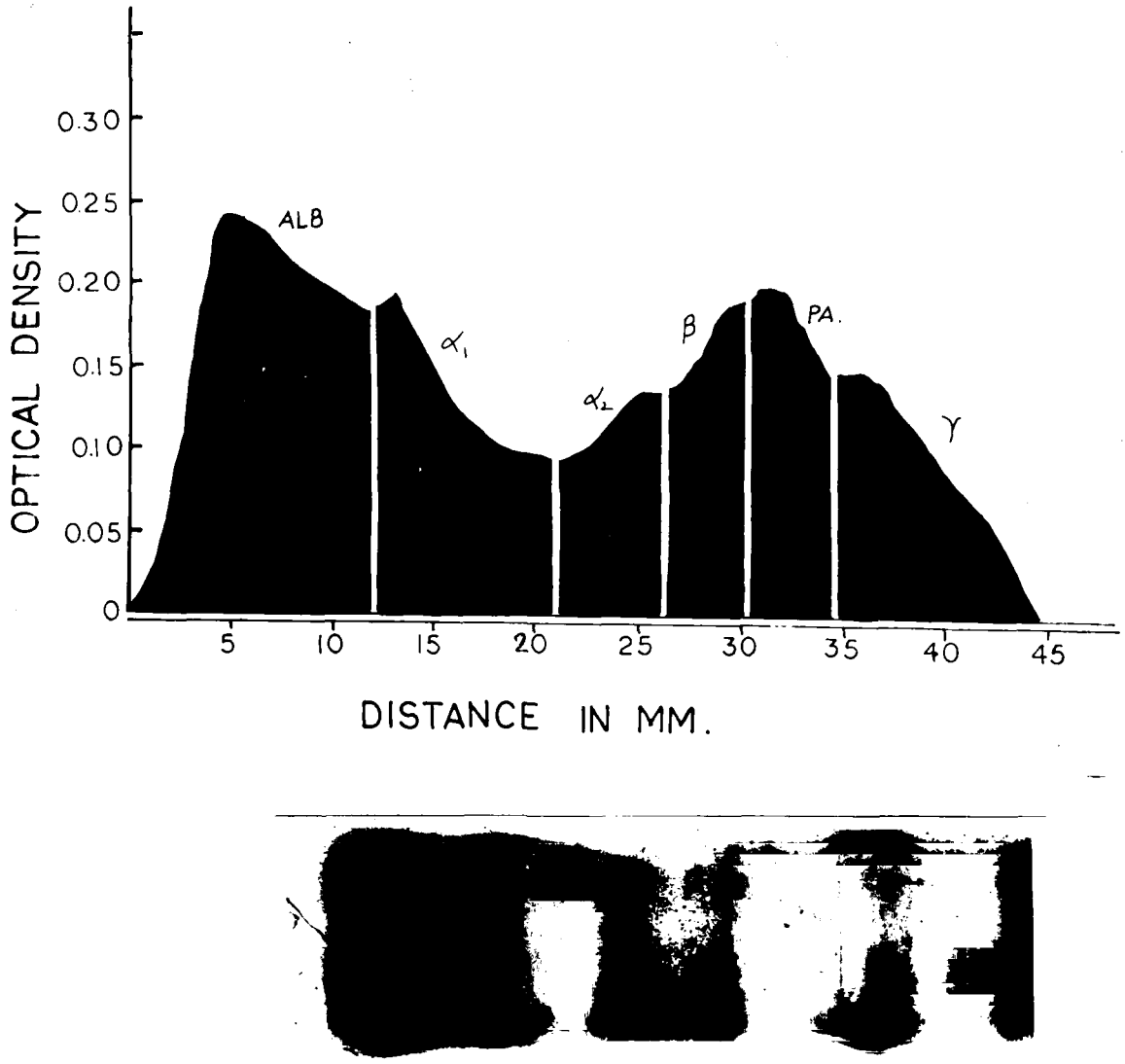


Fig. 6. Electrophoretic pattern of the infected rat serum (5th week).

6-28 days of initial infection.

In general, albumin level is higher than the globulin level in the serum of normal animals. But in most of the parasitic infections as reported by Stauber (1954) this has become reversed resulting into hyperglobulinemia accompanied by hypoalbuminemia. Such a condition has been reported in microfilariasis of buffaloes (Singh et al., 1972). The decrease in albumin, they reported, might be due to either a direct inhibiting effect in albumin production, a more rapid albumin catabolism, or an increased globulin concentration with consequent hypoalbuminemia. They have concluded that the diseases which produce liver lesions often cause a decrease in albumin level with relative increase in globulin level of blood. Hypoalbuminemia condition has also been reported by Berghen (1966) in pigeons following Capillaria infection, which he presumed to be due to either intestinal damage or poor appetite of infected birds resulting into a decrease in albumin and disturbance of osmotic equilibrium. Hypoalbuminemia condition has been reported to be undoubtedly a characteristic of liver involvement but, however, the hyperglobulinemia condition is a physiological compensation for the decreased albumin in order to maintain the blood's colloidal osmotic pressure is highly questionable (Gutmann, 1948).

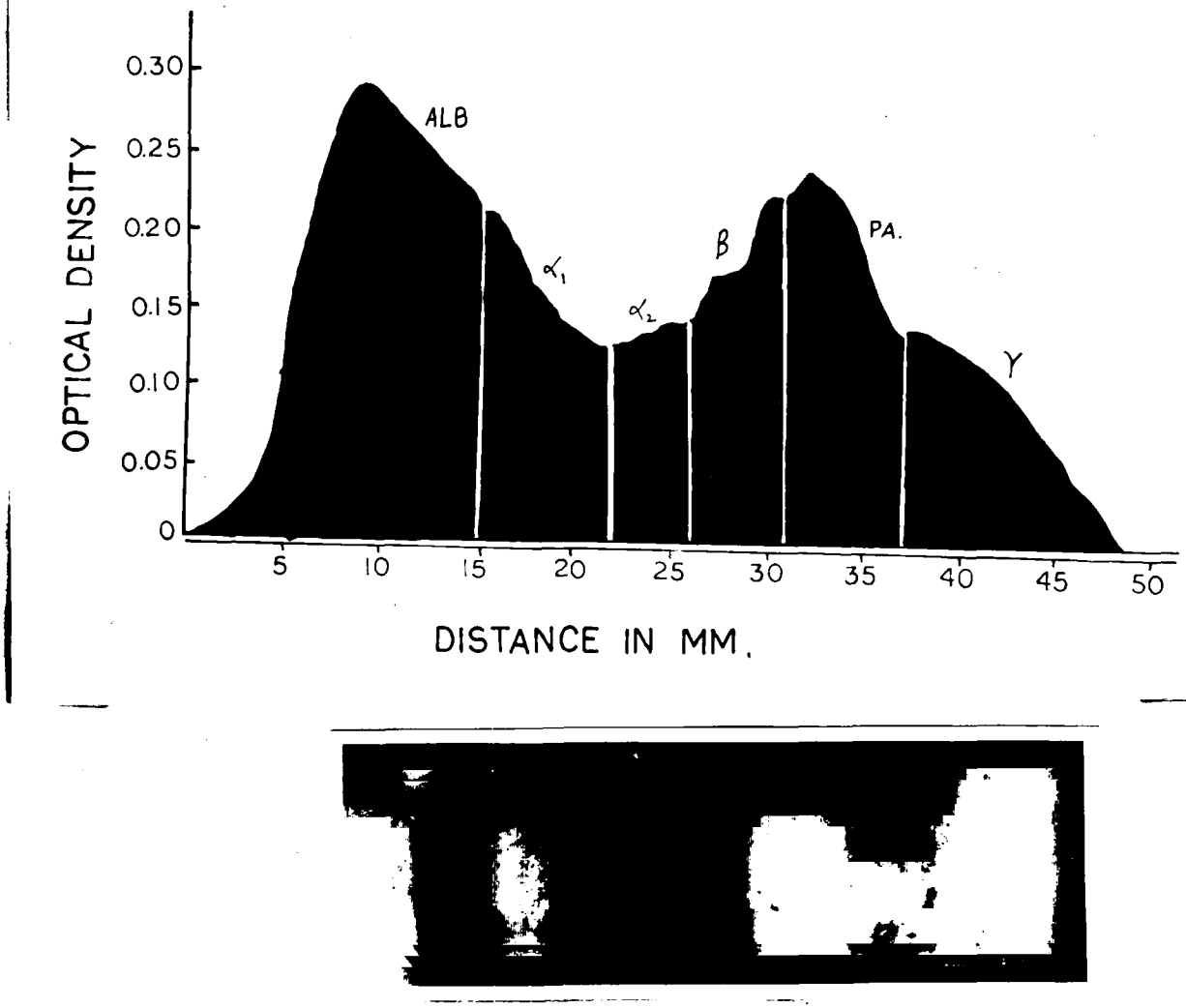


Fig. 7. Electrophoretic pattern of the infected rat serum (6th week).

Hawking and Thurston (1951), Ansari (1971) have reported that microfilariae accumulate in the liver, lungs and spleen etc. during their native phase, but it is not evident that their presence in these organs causes any pathology. However, the former authors have observed a thickening of alveolar walls, collapse and fibrosis in the lungs of monkeys and dogs infected with Mirofilaria, which they believed to be due to non-specific causes. The alterations in serum proteins in infected rats also could not be attributed to any sort of tissue injury caused by the surgical transplant because of two obvious reasons. First, the control rats treated in the same way did not show any change in serum proteins. Second, all the control and infected rats showed fast recovery and continued to be healthy. However, the tissue damage caused by embedding of adult worms in the peritoneal wall, mesentries and genital region might be responsible for a decrease in albumin and a compensatory increase in globulin levels. Since any attempt to work out the histopathology of the organs involved could not be undertaken, a definite conclusion would be premature at the moment.

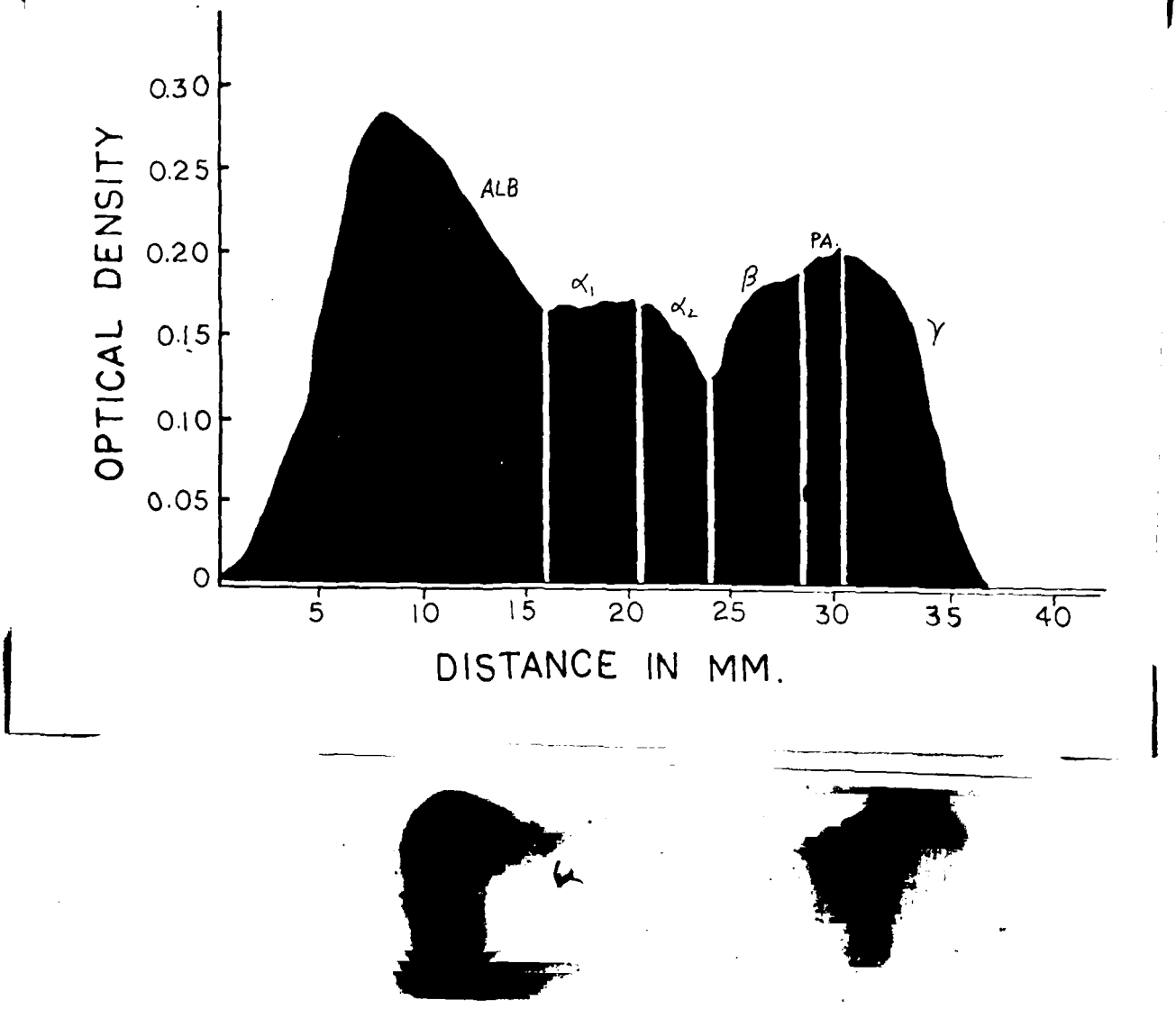
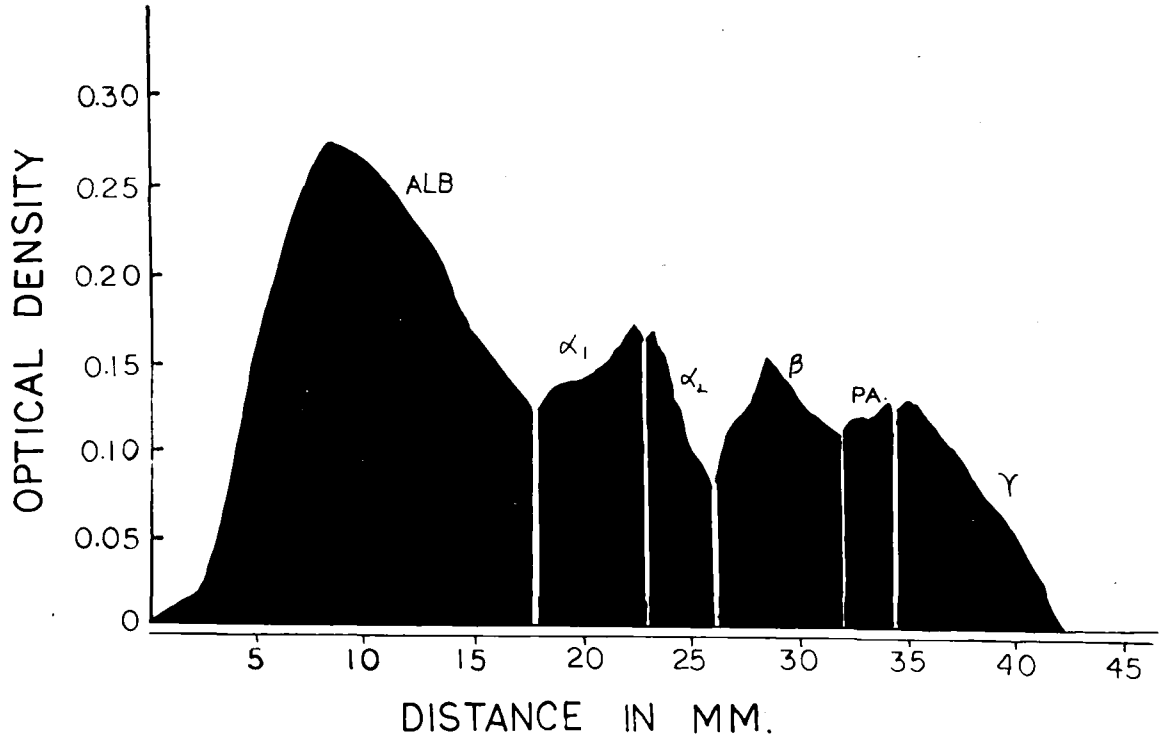


Fig. 8. Electrophoretic pattern of the infected rat serum (7th week).



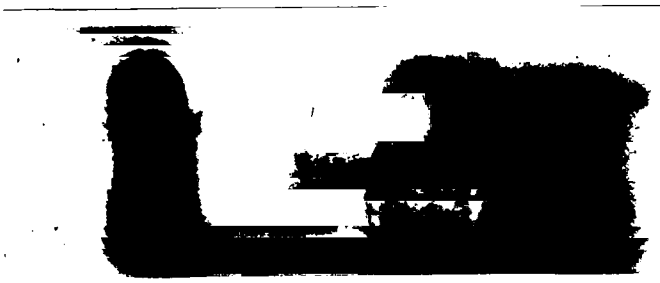


Fig. 9. Electrophoretic pattern of the infected rat serum (8th week).

Part B

LABORATORY REPORTS

I. PASSIVE CUTANEOUS ANAPHYLAXIS

INTRODUCTION

Nematode infections are known to induce the production of antibodies which are characterised by its ability to fix to the tissues. These antibodies are designated by a variety of terms : homocytotropic antibody, anaphylactic antibody, reagin-like antibody (Zwaifler et al., 1967; Ogilvie, 1964; Ivey, 1967; Bagni et al., 1966; Kobayashi and Sumi, 1973). Ogilvie (1964) suggested that reagin-like antibody might be responsible for immunity to helminths. This was confirmed by the same investigator later on (Ogilvie et al., 1966; Ogilvie, 1967; Jones and Ogilvie, 1967) through passive transfer of serum containing reagins to rats and its correlation with resistance in rats against Nippostrongylus and Schistosoma infections.

It is this property of skin fixation which provided an incentive to carry out this work.

MATERIALS AND METHODS

Antiserum

White rats were infected with Setaria cervi. In all 32 infected rats were used in this study. Microfilaria positive blood was collected directly by cardiac puncture without killing the animal, and serum was separated. Serum from normal rats served as control was also obtained. Weekly sample of sera collected from eight infected rats were pooled in 4 groups, and later used in passive cutaneous anaphylaxis test.

Preparation of antigen.

Antigen used in the experiment was phosphate buffered saline antigen and was prepared by the method of Nagai et al. (1968). Worms were first ground and defatted in chilled anhydrous ether, and later on extracted at 4°C for 48 hours in phosphate buffered saline (pH = 7.2). The mixture was centrifuged at 6,500 rpm for 30 minutes. Supernatant was used as antigen, and stored in a refrigerator after an addition of thiomersol (1:10,000) as a preservative. Protein content of antigen extract was estimated by the method described by Lowry et al. (1951). The total protein thus extracted was 1.25 mg/ml.

Passive cutaneous anaphylactic procedure.

Passive cutaneous anaphylactic (PCA) procedure used in the present study was essentially the same as described by Ivey (1967) and Bagni et al. (1968). White rats weighing from 150-200 gm and rabbits 1.5-2 kg were used in this experiment. A few hours before the experiment, the back of the animals was shaved. The method involved injections of 0.2 ml of antiserum and serum intradermally on the clean shaven backs of the rats and rabbits, and the injection sites were marked. About 4 hours and 48 hours later 250 µg of antigen (0.2 ml) was injected intradermally at the marked sites. Immediately after the antigen injection 0.5 ml of 1% Evans blue in saline was injected intracardially. The 'bluing reaction' occurring in the form of circular lesions at the site of injection within 30 minutes was measured, and the photograph of the underskin was taken.

EXPERIMENTAL DESIGN AND RESULTS

Immune rat sera were tested for homologous and heterologous PCA in white rats and rabbits respectively. There was no reaction in triplicate tests using rat immune sera in rabbits following 4- and 48-hour latent periods between antiserum and antigen injections. Positive reaction was observed only in white

rats using 4-hour latent period between antiserum and antigen injections. No positive reaction was observed in those rats when the interval between antiserum and antigen injections was 48 hours.

Non-specificity problem related to antigen concentration.

Four white rats, sensitised with normal rat serum, were challenged with varying concentrations of antigen i.e. 100 µg, 200 µg, 250 µg, 300 µg, 400 µg in order to find out the optimal dose. Each antigen concentration was used in 0.2 ml buffered saline. As saline control 0.2 ml of buffered saline was also introduced at two sites on each rat. A mean of eight measurements (2 per rat) of varying concentrations of antigen and saline was recorded. The results were analysed using Student's 't' test and are presented in Table I. Antigen concentration of 300 µg or higher provoked lesion sizes significantly larger than the control saline spot ($t = 6.44$, $P < 0.001$). Antigen concentration of 250 µg or lower produced lesion sizes similar to those provoked by saline solution alone, and they were not significantly different on statistical analysis. Hence antigen concentration of 250 ug in 0.2 ml was used as optimal dose throughout the test.

PCA test proper.

PCA test was done as described above with sera collected for 15 weeks of the initial infection. Weekly sample represented a pooled sera of 8 infected rats divided into 4 groups. Each group serum was used in 0.2 ml in dilutions of 1:10 to 1:640. Eight observations were made (2 per group/rat) on each week sera, and a mean of 8 lesion sizes and its corresponding antibody titers were recorded. PCA titer was recorded as the highest serum dilution giving a positive reaction (lesion size 7 mm or more in diameter). One antigen control, immune serum control and normal serum control were also included which showed lesion sizes varying between 3.7 to 5.5 mm throughout the experiment. The lesion sizes of immune sera were larger consistently than all control groups (Table II, Plate 1).

Table II shows the results of the weekly changes in PCA titers, and mean diameter lesions of immune sera as compared to control group. It also indicates a comparison of results in lesion sizes between an immune serum and normal serum control. The mean lesion size of an immune serum was significantly larger than normal serum control lesion ($t = 7.45$, $P < 0.001$).

Weekly test for PCA activity in rats showed the positive reaction in the 2nd week of the initial infection with a mean PCA titer of 1/40, thereafter PCA titer began to rise and reached

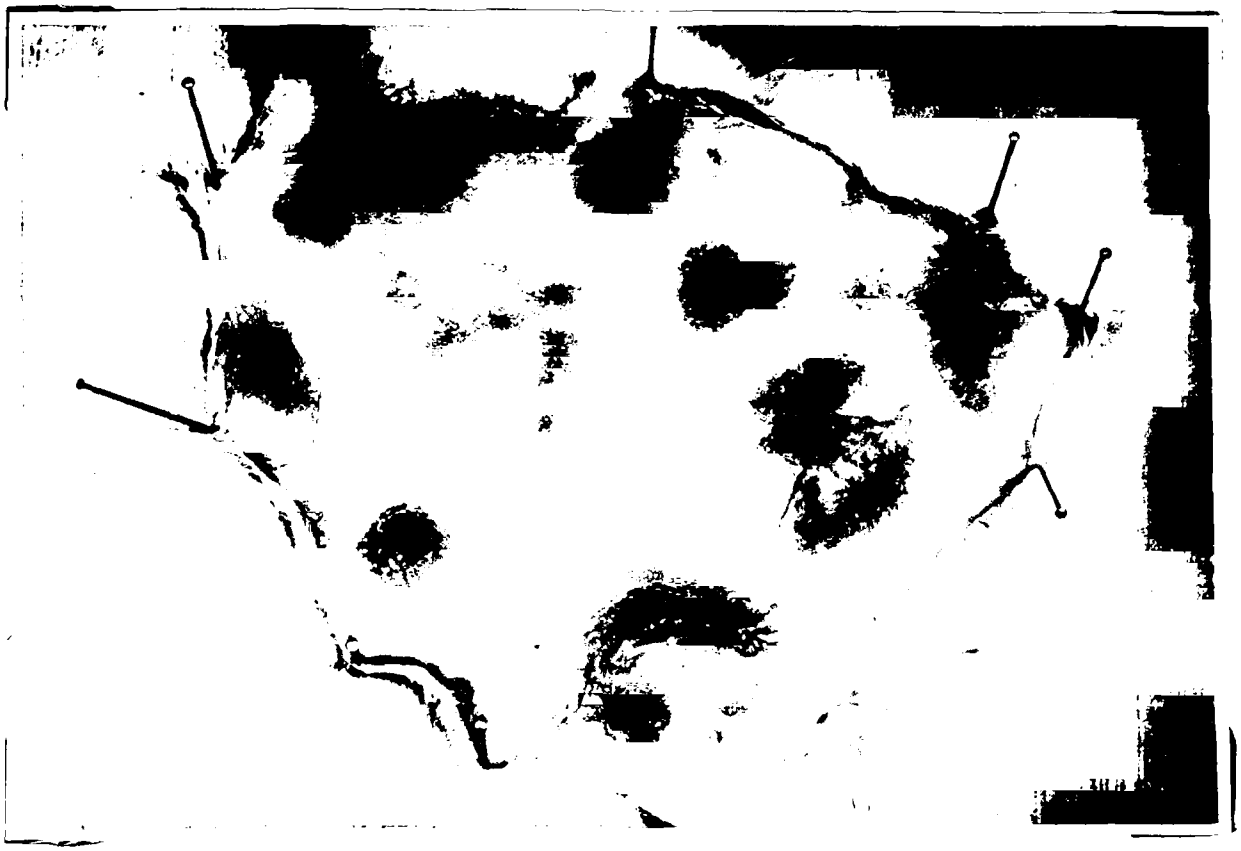


Plate 1. Rat skin showing PCA reaction. Larger lesions showing positive reaction and smaller ones as negative control groups.

Table I. The diameter of the lesion sizes resulting from an injection of *Salaria servi* antigen or saline into white rats sensitised with normal rat serum.

Test material	White rats sensitised with normal serum					
	Antigen in 0.2 ml					Saline
	100 µg	200 µg	250 µg	300 µg	400 µg	0.2 ml
No. of observations	8	8	8	8	8	8
Mean diameter of lesions (mm)	4.0	4.0	4.25	5.75*	6.1	3.87*
S.E. of mean	0.26	0.29	0.29	0.16	0.21	0.26

* Compared

$t = 6.44$

$P < 0.001$

Table II. Mean lesion sizes resulting from an injection of Listeria cervi antigen into mice sensitized 4 hours previous with test materials and its corresponding PCA titer.

Weeks after infection	Mean lesion size of immune serum ± SE (mm)	PCA titer of immune serum	Mean lesion size of immune serum control ± SE (mm)	Mean lesion size of normal serum control ± SE (mm)	Mean lesion size of antigen control ± SE (mm)
1	6.0 ± 0.26	0	5.2 ± 0.21	5.15 ± 0.26	4.2 ± 0.29
2	*9.1 ± 0.24	40	4.6 ± 0.37	*5.30 ± 0.19	3.9 ± 0.51
3	9.4 ± 0.37	85	4.9 ± 0.32	5.30 ± 0.26	4.3 ± 0.32
4	10.2 ± 0.45	100	5.3 ± 0.24	5.50 ± 0.31	4.0 ± 0.24
5	12.6 ± 0.55	160	5.5 ± 0.22	4.80 ± 0.51	3.8 ± 0.39
6	12.2 ± 0.67	160	5.0 ± 0.29	5.00 ± 0.19	4.3 ± 0.33
7	11.5 ± 0.41	85	5.3 ± 0.16	5.30 ± 0.17	4.4 ± 0.41
8	10.1 ± 0.37	80	4.6 ± 0.30	5.40 ± 0.22	4.6 ± 0.25
9	9.7 ± 0.31	80	5.1 ± 0.41	4.60 ± 0.65	4.1 ± 0.47
10	9.5 ± 0.37	80	5.3 ± 0.21	4.70 ± 0.41	4.2 ± 0.41
11	9.2 ± 0.29	40	4.4 ± 0.26	4.90 ± 0.37	4.1 ± 0.43
12	8.0 ± 0.31	15	4.6 ± 0.47	5.10 ± 0.41	3.7 ± 0.23
13	8.0 ± 0.25	10	5.1 ± 0.32	5.50 ± 0.18	4.3 ± 0.23
14	7.7 ± 0.33	10	4.5 ± 0.24	4.90 ± 0.41	3.9 ± 0.66
15	5.6 ± 0.25	0	4.5 ± 0.21	4.60 ± 0.66	4.0 ± 0.33

* Compared

$t = 7.45$

its peak between 5th and 6th weeks of the initial infection (Table II, Fig. 1). PCA titer was recorded at high level from 4 to 6 weeks of initial infection and thereafter showed a declining tendency. By the end of 15th week PCA titer became undetectable.

DISCUSSION

In general homocytotropic antibodies have been reported to be efficiently produced by most mammalian hosts following nematode infections. Ogilvie et al. (1966), Jones and Ogilvie (1967) have demonstrated protective role of such antibodies (reagin-like) in rats against Nippostrongylus and Schistosoma infections.

In the present study homocytotropic antibody was detected in the serum after a week of initial infection and persisted for 14 weeks attaining its peak by 5th and 6th weeks of infection. It all points out to the fact that homocytotropic antibody may be evoked by living worms only. It has been observed that adult worms implanted into the peritoneal cavity of rats normally survived for about 4-6 weeks, and thereafter started disintegrating. Microfilariae have been found to persist in the blood for a much longer duration (65 ± 5 days) but no correlation between

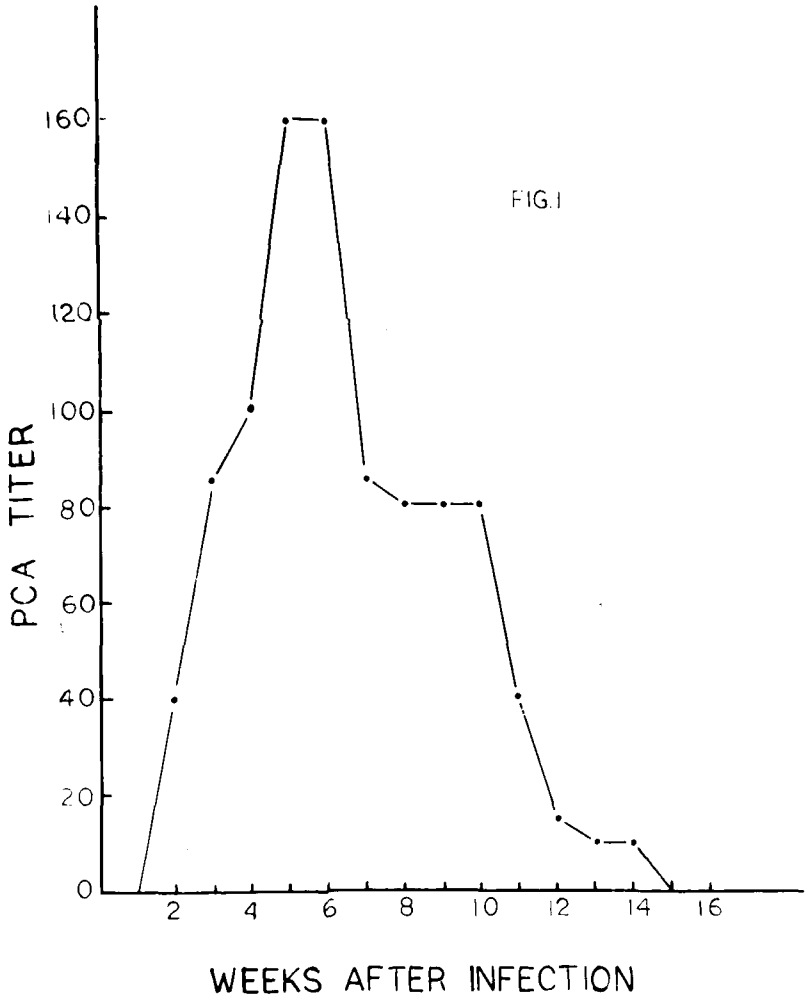


Fig. 1. Mean PCA titer obtained in white rats infected with B. cervi following short sensitization period.

PCA titer and level of microfilaraemia was evident. Kobayashi and Sami (1973) have also reported that there was a direct relationship between live worms and homocytotropic antibody production in rabbits against Mirofilaria infection - there was a gradual decrease in PCA titer where live adult worms were removed from rabbits on 27th day after implant.

It is now established that PCA is mediated by two different kinds of antibodies. In the first, serum from one species produces PCA reactions in the skin of another species e.g. rabbit serum in guinea pig skin. This type of reaction, is known as heterologous anaphylaxis or heterocytotropic anaphylaxis. This type of anaphylactic reaction has been described by Nagai et al. (1968) who reported that serum from rabbits infected with Litomosoides carinii produced PCA reactions in the guinea pig skin. The second kind of anaphylactic reaction is known as homologous anaphylaxis or homocytotropic anaphylaxis. In this reaction anaphylactic antibody gives PCA reactions in the skin of the same species from which the antibody is derived. This type of anaphylactic reaction has been described by Sadun et al. (1967), Nagai et al. (1968), Mota et al. (1969) in rabbits, rats and mice in response to infections with Mirofilaria, Litomosoides and Schistosoma respectively.

In the present study white rats were found to be producing only homocytotropic antibody or homologous anaphylactic

antibody against Setaria cervi. Consequently, when rat sera containing anaphylactic antibody was introduced into the skin of rabbit did not show PCA reaction. This may be due to species difference as earlier pointed out by Nagai et al. (1969) in rats infected with Litomosoides carinii. Similarly, Kota et al. (1969) reported that PAC activity in mouse - L. ranagni sera was poorly transferable to rats and not at all to guinea pigs.

Recent studies have shown that two kinds of anaphylactic antibodies are produced - one which gives PCA reactions induced after a short sensitization period (2-4 hours) and second which is responsible for PCA reactions induced after long sensitization period (40-72 hours). The present study shows that white rats produce the sort of anaphylactic antibody which is detectable by PCA test after short sensitization period (4 hours). No positive PCA reaction was observed using 48-hour latent period between antiserum and antigen challenge. These observations are in accordance with earlier investigators such as Kota (1966). Kota et al. (1969) who reported that infection of mice with Schistosoma produced homocytotropic antibody detectable by PCA using a short sensitization period of 3 hours; no such antibody was detectable in the same animal by PCA using a long sensitization period of 72 hours. Kota (1968) reported that homocytotropic antibody in mice responsible for PCA reaction

induced after short sensitization period could be 7 S γ 1 globulin. Reports are also available which indicate that mice and dogs are able to produce two types of skin sensitizing antibodies - one detectable by PCA after short sensitization period (4 hours) and another after long sensitization period (48-72 hours) following infections with Trichinella and Microfilaria respectively (Kota et al., 1969; Hsu et al., 1974). The homocytotropic antibody induced after long sensitization period has been reported to be reagin-like antibody (Kota, 1968).

II. COMPLEMENT FIXATION TEST

INTRODUCTION

Complement fixation test has been considerably used in the serodiagnosis of nematode infections specially in filarial infections (Warren et al., 1946; Manning and McMadsenn, 1956; Soulsby, 1956; Schofield, 1957; Rosseau-Vanalde and Janssens, 1961; Taffs, 1964; Tanaka et al., 1969; Zahner, 1974). The test has been reported to be sensitive in detecting complement fixing antibodies against a given parasite.

The principle involved in complement fixation test (CFT) points out that if antibodies against a given parasite are present in the serum, these will react with corresponding antigen in the presence of complement, and as a result complement will be used up in the antigen-antibody system. It is consequently not available to hemolyse erythrocytes which are subsequently added. The absence of lysis proves, therefore, the presence of antibodies i.e. complement fixation test is positive.

In view of the specificity of the test present study was carried out in white rats to ascertain complement fixing antibodies against Setaria cervi infection. Entire experimental procedure was followed after the classical Kolmer's technique (Kolmer et al., 1951).

MATERIALS AND METHODS

Preparation of antisheep hemolysin (Amboceptor).

For antisheep hemolysin, sheep blood was collected from abattoir in Alsever's solution. The blood was collected in Alsever's solution in equal volume and thoroughly mixed. Later, the suspension was filtered through cotton and was stored in a refrigerator. For use, a required amount of the blood suspension was centrifuged at 2500 rpm. The supernatant was removed and the corpuscles left over were washed twice with normal saline. Thoroughly washed and packed sheep corpuscles were mixed with equal volume of normal saline. Four rabbits were inoculated with 1.5 ml of the cell suspension intravenously through marginal ear vein. In all six injections were given to each rabbit at 5-day interval. Bleedings were made 7-9 days after the last injection. Separated sera were pooled and titrated for hemolysin content. Hemolysin titration as given in Table I showed amboceptor titer in rabbit sera as 1:2000. Pooled rabbit

serum was preserved by the addition of equal volume of best grade neutral glycerine, and kept in a refrigerator for later use.

Hemolysin titration.

A stock dilution of 1:100 hemolysin was prepared by mixing 2 ml of glycerinised hemolysin with 94.0 ml normal saline and 4.0 ml of 5% phenol in normal saline. From this stock dilution a series of higher dilutions ranging from 1:500 to 1:5000 were prepared and used in 0.5 ml amount as shown in Table I. Hemolysin titration was done against 0.3 ml of 1:30 dilution of guinea pig complement and 2% sheep cells prepared in normal saline. Blood was collected from a number of guinea pigs through cardiac puncture and serum thus obtained was used as a source of complement.

The contents of the tube were mixed well and incubated in water bath at 37°C for 1 hour. Centrifuged lightly and noted the degree of hemolysis in spectrophotometer at a wave-length of 530 mμ. Highest dilution of hemolysin that gave apparently complete sparkling hemolysis and optical density range of 0.620-0.699 was taken as amboceptor unit. Complete hemolysis of 0.5 ml of 2% sheep cells mixed with 2.5 ml distilled water gave an optical density range of 0.620-0.699.

Table I. Hemolysin titration

Tube	Hemolysin (0.5 ml)	Complement 1:30 (ml)	Normal saline (ml)	2% sheep cells (ml)	Results
1	1 : 500	0.3	1.7	0.5	Complete hemolysis
2	1 : 1000	0.3	1.7	0.5	Complete hemolysis
3	1 : 1500	0.3	1.7	0.5	Complete hemolysis
4	1 : 2000	0.3	1.7	0.5	Complete hemolysis
5	1 : 3000	0.3	1.7	0.5	Partial hemolysis
6	1 : 4000	0.3	1.7	0.5	No hemolysis
7	1 : 5000	0.3	1.7	0.5	No hemolysis
8 (control)	1 : 500	Nil	2.0	0.5	No hemolysis
9	-	-	2.5 (distilled water)	0.5	Complete hemolysis

and 0.655 as an average figure. Table I showed amboceptor unit as 1:2000. In all complement titration, antigen titration and main CFT two units of this amboceptor in 0.5 ml amount was used.

Complement titration.

In the preliminary test the complement quantity was determined which was just sufficient to guarantee lysis of the sheep red cells through 2 units of amboceptor amount. Various dilutions of 1:30 complement were prepared in a series of tubes as shown in Table II.

The smallest amount of the complement just giving complete sparkling hemolysis was the exact unit. Table II shows 0.6 ml of complement as the exact unit, and the next higher tube is the full unit. To be on the safe side in conducting the antigen titration and main CFT 2 full units of complement contained in 1 ml amount was employed.

Preparation of antigen.

Table II. Titration of complement

Tube	Complement 1:30 (ml)	Normal saline (ml)		Hemolysin 2 units (ml)	2% sheep cells (ml)		Results
1	0.1	1.9	Incubated at 37°C for 1 hour	0.5	0.5	Incubated at 37°C for 1 hour	No hemolysis
2	0.2	1.8		0.5	0.5		No hemolysis
3	0.3	1.7		0.5	0.5		No hemolysis
4	0.4	1.6		0.5	0.5		No hemolysis
5	0.5	1.5		0.5	0.5		Partial hemolysis
6	0.6	1.4		0.5	0.5		Complete hemolysis
7	0.7	1.3		0.5	0.5		Complete hemolysis
8	0.8	1.2		0.5	0.5		Complete hemolysis
9 (control)	Nil	2.0		0.5	0.5		No hemolysis

ground to pieces with glass tissue grinder in sufficient amount of cold ether. Ether was drained off and the ground material was desiccated at 37°C for 24 hours. The dry powdered material was treated with 50 ml of cold acetone, and homogenised in a tissue grinder. The mixture was filtered, and the supernatant was discarded. The process was repeated again. Acetone treated material was again dried. The dry powder was weighed, suspended in PBS (1 gm dry powder in 100 ml PBS) and allowed to extract in a refrigerator for 48 hours with occasional stirring. The mixture was then centrifuged at 6,500 rpm for 30 minutes. The supernatant was used as antigen. To avoid bacterial growth thiomersol (1:10,000) was added to the antigen and kept in a refrigerator.

Titration of antigen.

The anticomplementary activity of the antigen was determined against 2 full units of complement and hemolytic system: the results are presented in Table III. Complete hemolysis took place at an antigen dilution of 1:40. To obviate non-specific reactions in the test, the antigen was arbitrarily used in a dilution of 1:80 in 0.5 ml amount. This strength of antigen was used throughout the CFT.

Table III. Titration of antigen

Tube	Antigen (0.5 ml)	Complement 2 full units (ml)		Hemolysin 2 units (ml)	2% sheep cells (ml)		Results
1	1 : 5	1.0	Incubated at 37°C for 1 hour	0.5	0.5	Incubated at 37°C for 1 hour	No hemolysis
2	1 : 10	1.0		0.5	0.5		No hemolysis
3	1 : 20	1.0		0.5	0.5		No hemolysis
4	1 : 40	1.0		0.5	0.5		Complete hemolysis
5	1 : 80	1.0		0.5	0.5		Complete hemolysis
6	1 : 160	1.0		0.5	0.5		Complete hemolysis
7 (control)	1 : 5	Nil		0.5	0.5		No hemolysis
8 (control)	(normal saline)	1.0		0.5	0.5		Complete hemolysis

Complement fixation test

Weekly sera from 12 infected rats were collected and pooled. The pooled antiserum diluted in 1:5 to 1:640 was used in 0.5 ml amount. At first antiserum was incubated at 56°C for 30 minutes to remove anticomplementary substance since the result of the test depended on the quantity of free complement. In this test immune serum control, antigen control, normal serum control and hemolytic control were also included. The results of CFT are presented in Table IV.

A positive reaction or end point was taken as the one which gave 50% hemolysis, and hemolysis above 50% was taken as negative results. Plate 1 shows the main CFT. +++ indicates complete inhibition of hemolysis (positive reaction), ++ indicates 50% hemolysis and(-) shows complete hemolysis (negative result). Last tube shows inhibition of hemolysis (control tube) in the absence of complement. Rest of the tubes showing complete hemolysis are immune serum control and standard lysate (0.5 ml of 2% sheep cells + 2.5 ml distilled water).

The percentage of hemolysis of the test solutions was calculated according to the formula:

$$\text{Hemolysis (\%)} = \frac{ODa}{ODb} \times 100$$

in which ODa is the optical density of the test solution and

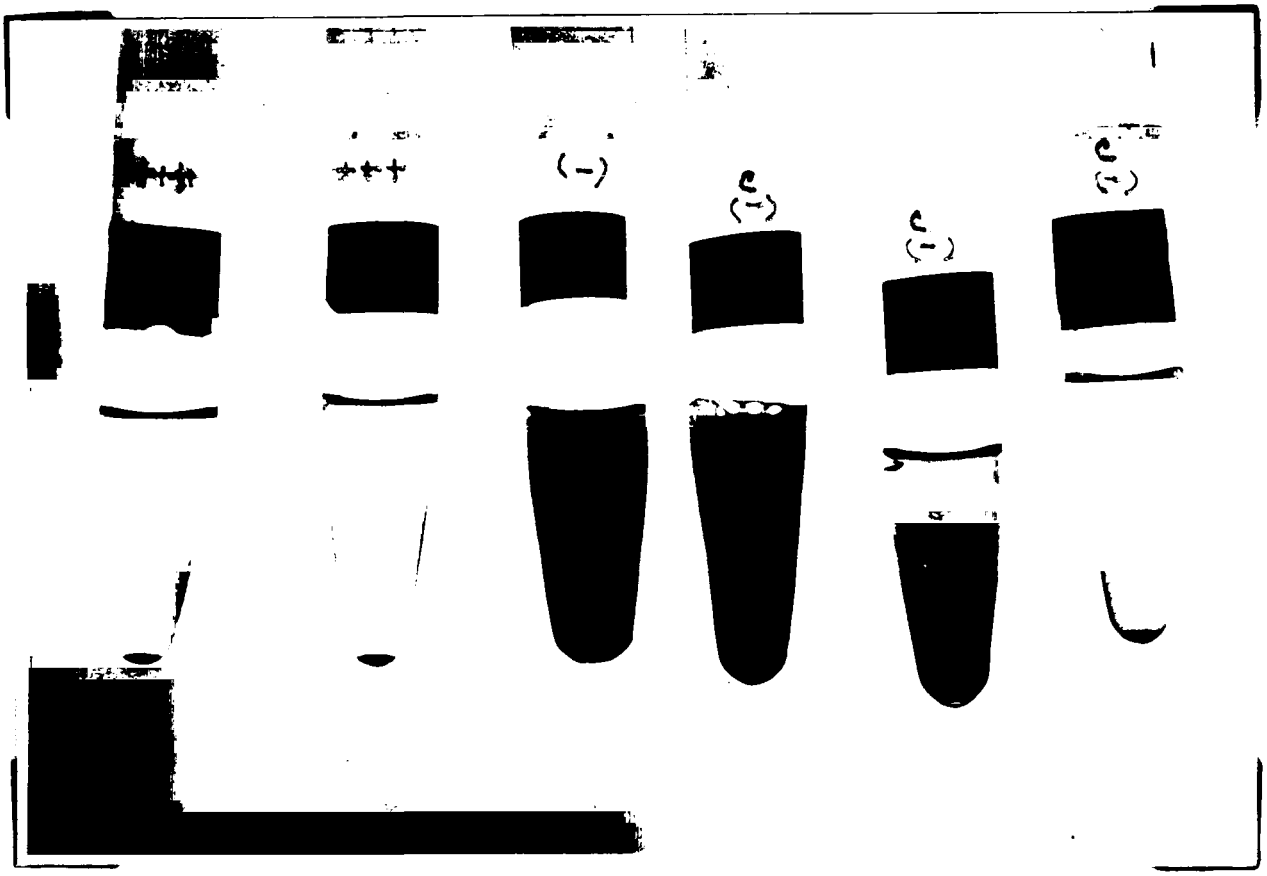


Plate 1. Complement fixation test. +++ and ++ indicate positive reaction, (-) indicates negative reaction and C (-) indicatesvarious negative controls.

Table IV. Complement fixation test

Tube	Antiserum (0.5 ml)	Antigen 1:30 (ml)	Complement 2 full units 1:30 (ml)	Overnight refrigeration followed by incubation at 37°C for 1 hour	Hemolysin 2 units (ml)	2% sheep cells (ml)	Incubated at 37°C for 1 hour	Results
1	1 : 5	0.5	1.0		0.5	0.5		No hemolysis
2	1 : 10	0.5	1.0		0.5	0.5		No hemolysis
3	1 : 20	0.5	1.0		0.5	0.5		No hemolysis
4	1 : 40	0.5	1.0		0.5	0.5		No hemolysis
5	1 : 80	0.5	1.0		0.5	0.5		Partial hemolysis
6	1 : 160	0.5	1.0		0.5	0.5		Complete hemolysis
7	1 : 320	0.5	1.0		0.5	0.5		Complete hemolysis
8	1 : 640	0.5	1.0		0.5	0.5		Complete hemolysis
9 (control)	1 : 5	Nil	1.0		0.5	0.5		Complete hemolysis
10 (control)	Nil	0.5	1.0		0.5	0.5		Complete hemolysis
11 (control)	1 : 5	0.5	Nil		0.5	0.5		No hemolysis
12 (control)	1 : 5 (normal serum)	0.5	1.0		0.5	0.5		Complete hemolysis
13	2.5 ml (distilled water)	-	-		-	0.5		Complete hemolysis

ODb is the optical density of lysate from 0.5 ml of sheep RBC plus 2.5 ml distilled water which gave an average OD value 0.655. In all control tubes negative results were noted.

RESULTS

Table IV presents the results of complement fixation test using infected rat sera. Complement fixing antibody was detected in the 2nd week of initial infection with the titer of 1:5. The antibody titer rapidly increased and reached its maximum between the 4th and 5th weeks with titers of 1:80 and 1:40 respectively. A fall in antibody titer, which followed the initial rise, occurred within two weeks with titers of 1:10. No antibody titer was detected during 8th week of initial infection (Fig. 1). After the disappearance of antibody in primary infection, a reinfection of 5 females and 2 males was given in some rats and a record of antibody titer was made for 3 weeks. It was observed that the rats responded immediately in challenge infection and a high titer of 1:20 was obtained in Ist week which maintained in 2nd week also. In the 3rd week titer rose to 1:80.

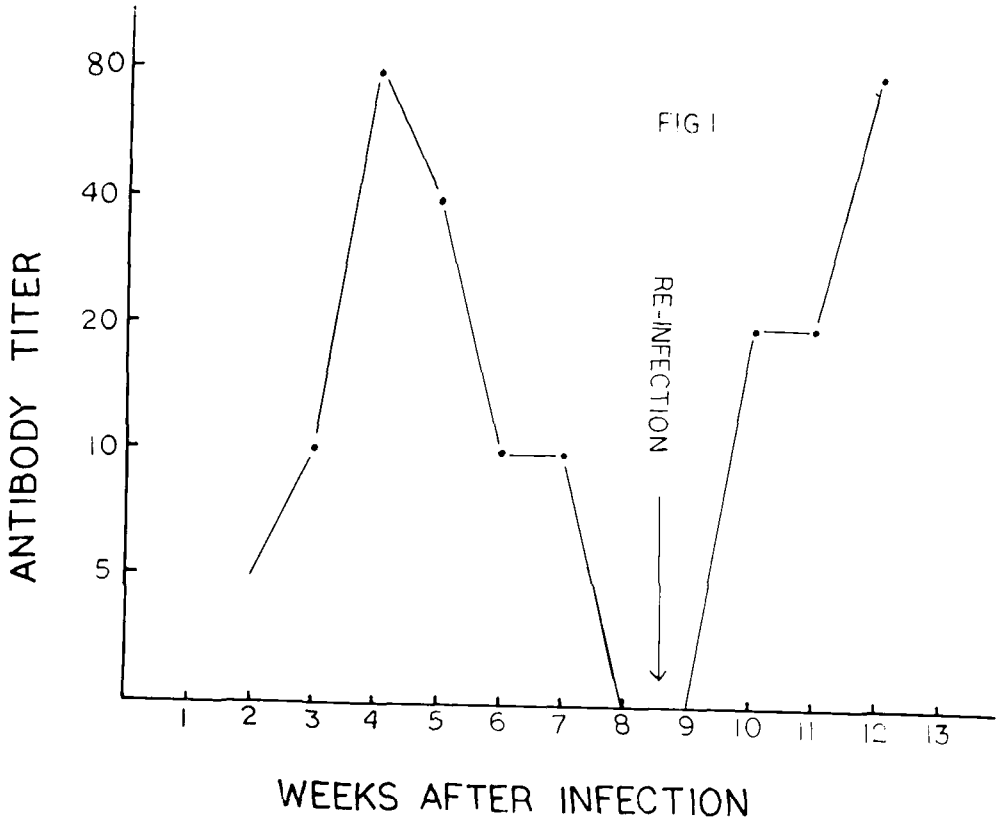


Fig. 1. The antibody response in white rats infected with S. cervi. Complement fixation test.

DISCUSSION

Complement fixing antibodies were detected in the serum of infected rats during 2nd week until 7th week with a maximum titer of 1:80 during 4th week. The rise and fall of antibody titer has been found to coincide with the maximum microfilarial concentration during 3rd and 4th weeks and survival of adult worm in the peritoneal cavity of rats (4-6 weeks). A definite correlation between the worm burden and complement fixing antibody titer was also reported by Tanaka et al. (1969) in Litomosoides carinii in cotton rats. Similarly, Zahner (1974) detected complement fixing antibody in Litomosoides infection showing significant correlation with the number of circulating microfilariae in the blood. Pacheco (1966) reported high level of antibody titer in dirofilariasis of dogs during the period of active migration of larvae.

In another study Taffs (1964) detected complement fixing antibodies in Ascaris infection in rabbits as early as 5-8 days and reached its maximum between 8-21 days after initial infection. By the end of 33 days no antibody was detected. He further reported a definite relationship between the infective dose and antibody response. The larger the initial dose, the greater the antibody response, and the longer the period for which antibody could be detected.

In the present study a high level of antibody response was recorded in secondary infection as compared to the primary infection. This could be attributed to the slightly large dose of the parasite in secondary infection. These observations are in accordance with the earlier report made by Taffs (1964) in experimental infection of *Ascaris suum* in rabbits.

III. PRECIPITIN TESTS

Precipitin tests have been widely employed for a variety of immunological investigations particularly in the diagnosis of filarial infections. Precipitin ring test has been reported to be specific and sensitive. Culbertson *et al.* (1944a) reported that the test had a sensitivity equal to the complement fixation test. Okabe *et al.* (1957, 1961) in a series experiments found the test to be highly specific in diagnosis of wuchereriasis. Okabe and Ono (1957) also found it highly sensitive and specific in the diagnosis of experimental setariasis in rabbits. Kagan and Bargai (1956) used the precipitin test in the diagnosis of trichinosis. Gaur and Dee (1972) obtained positive results in ascariasis utilising precipitin ring test.

Precipitin ring test involves the principle of free mixing of homologous antigen-antibody system. If the sera is positive a visible ring is formed at the interface of antigen and antiserum. Unlike other serologic tests, antigen in this test is used in serial dilutions against full strength of serum. The end point or titer is represented by the positive results obtained against highest antigen dilution. This method does not suffer greatly from inhibition by antigen excess because diffusion

at the interface provides a zone of nearly optimal proportions in which precipitation occurs (Carpenter, 1965).

Precipitation of antigen-antibody system has also been studied in agar medium. Unlike precipitin test, antigen and antiserum are allowed to diffuse in agar medium. This method permits the determination of the minimum number of antigenic components present in the antigen extract. The method was described for the first time by Oakley and Fulthorpe (1953) and later a modification known as Freer technique (Freer, 1956) was employed by Kagan and Bargai (1956) for serodiagnosis of trichinosis.

EXPERIMENTAL PROCEDURE

Antigen.

Antigen used in precipitin ring test and double diffusion test was Melcher's antigen which was prepared as follows. Dried powdered worm weighing 250 mg was extracted in petroleum ether at 52°C for one week. The material was mixed with 20 ml of borate buffer (pH = 8.2) and stored at 4°C for 24 hours. Later, the material was centrifuged and sediment was discarded. pH of the supernatant was adjusted to 4.8 with 0.2 N HCl. The acid insoluble precipitate which came out of the solution at the low pH was separated by centrifugation and discarded.

The clear supernatant was used as antigen in the experiments.

Antiserum.

Antiserum came from rats infected with Setaria servi. Weekly samples of pooled antiserum of 10 rats were obtained.

Precipitin ring test.

Precipitin ring test was performed according to the method described by Kagan and Bargai (1956) and Carpenter (1965). Antiserum in 0.2 ml amount was placed in precipitin tubes which was overlaid with the same amount of antigen. Antigen was expelled carefully with the help of tuberculin syringe so that it run slowly down and formed a layer over the serum. Titrations were made with the various dilutions of antigen prepared in saline against full strength of serum. Normal serum control, antiserum control and antigen control were also included. Tubes were incubated at room temperature for 24 hours followed by overnight refrigeration. Positive reactions were taken as those forming a visible ring at the interface of antigen and antiserum. The titer was indicated by the reciprocal of the highest antigen dilution that gave a detectable reaction.

Double diffusion.

Double diffusion test for the antigenic analysis of Melcher's antigen was made after the technique of Kagan and Bargai (1956) and Carpenter (1965). 0.1 ml serum was placed

in the bottom of the precipitin tube and 0.1% agar in phosphate buffer (pH = 7.0) was carefully overlaid and allowed to solidify. 0.1 ml of antigen was then added as the top layer. The tubes were incubated for 48 hours at room temperature. One normal serum control was also included in which normal serum was overlaid with antigen. Planes of precipitate appeared in the agar layer where antigen and antibody met in serologically equivalent proportions.

RESULTS

Precipitin ring test was positive from Ist week of initial infection. Titration of antigen against antiserum showed increasing level in the titer as the infection progressed. Serum taken from 3rd to 5th weeks after infection was positive with antigen dilution of 1:400 (Fig. 1). During the 6th week antigen titer was 1:100. Serum taken 49 days after infection was negative even at the lowest dilution of antigen. Plate 1 shows visible ring (positive reaction) at an antigen dilution of 1:400 and 1:100 during 4th week. The three remaining tubes are various controls showing negative reactions.

Double diffusion in agar showed positive reaction to serum collected 28 days of initial infection. Three bands were seen in infected serum (Plate 2). No bands were observed in normal control rat serum.

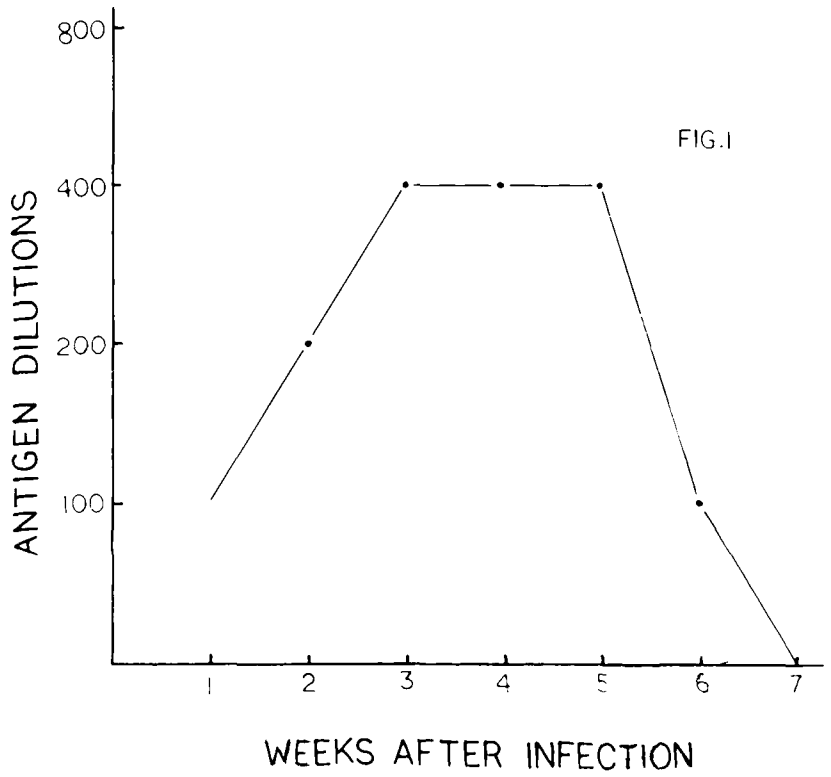


Fig. 1. Titer of Melcher's antigen in precipitin ring test with rat serum infected with S. cervi.

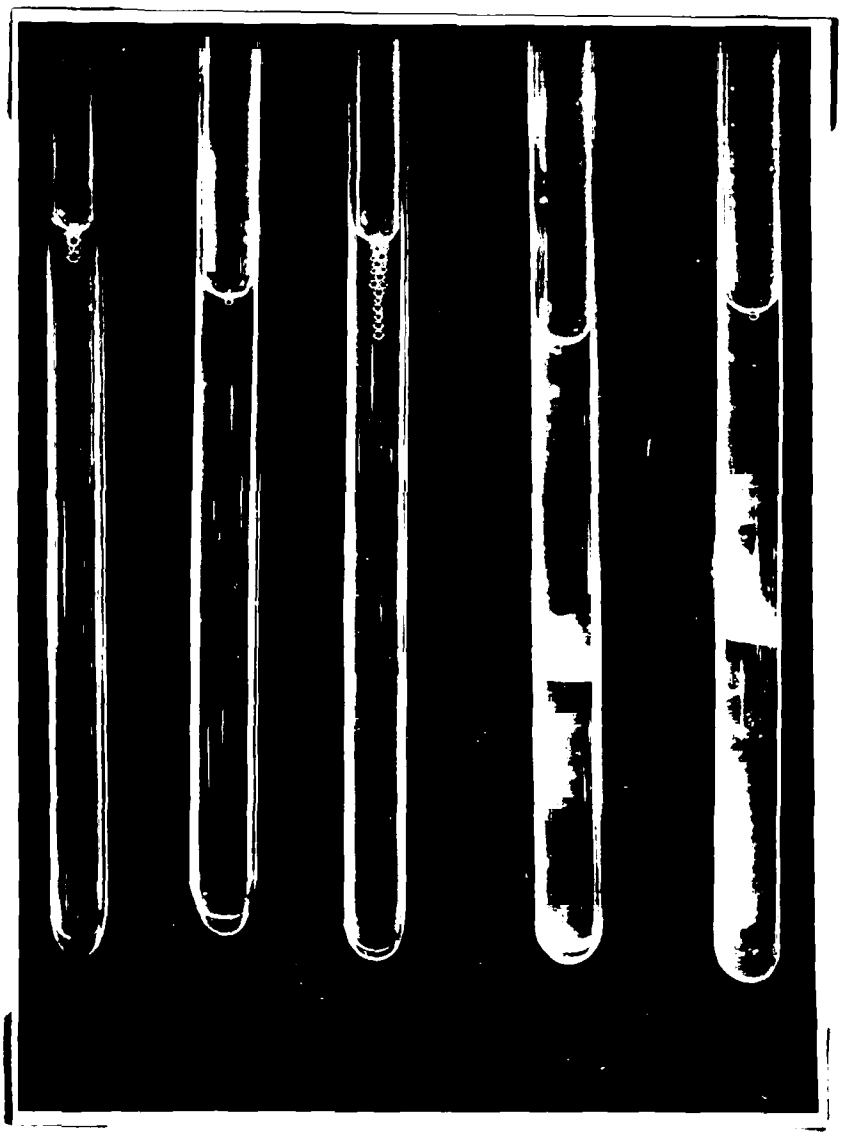


Plate 1. Precipitin ring test. The two tubes at the right showing positive reaction at an antigen dilution of 1:100 and 1:400, and the remaining tubes are various controls showing negative reaction.

DISCUSSION

For detection of antibody in infected sera and determination of minimum number of components present in a given antigenic mixture precipitin ring test and double diffusion in agar have been reported to be quite specific and sensitive (Okabe and Ono, 1957: Carpenter, 1965). Working on experimental infection of *Setaria equina* in rabbits Okabe and Ono (1957) reported very high levels of titers detectable through precipitin ring test. The high titers, they reported, were maintained from 6 days to 31 days after initial infection and then declined. Similarly, in another study Okabe *et al.* (1957) using *Setaria equina* and *S. cervi* antigen observed positive precipitin tests using oxen sera infected with *Onchocerca gutturosa* and *Setaria cervi*. They reported that *Setaria equina* antigen was comparatively more sensitive than that of *S. cervi* because of the fact that precipitin test was positive using *S. equina* antigen even at a dilution of more than 1:400.

In the present study precipitin test was found to be positive from 7 days and remained so until 42 days. It points out that the precipitin antibody appears in this infection early. The present findings are also in accordance with earlier investigators (Okabe *et al.*, 1957) who showed that precipitin antibody by *S. cervi* are induced in a short time after infection.

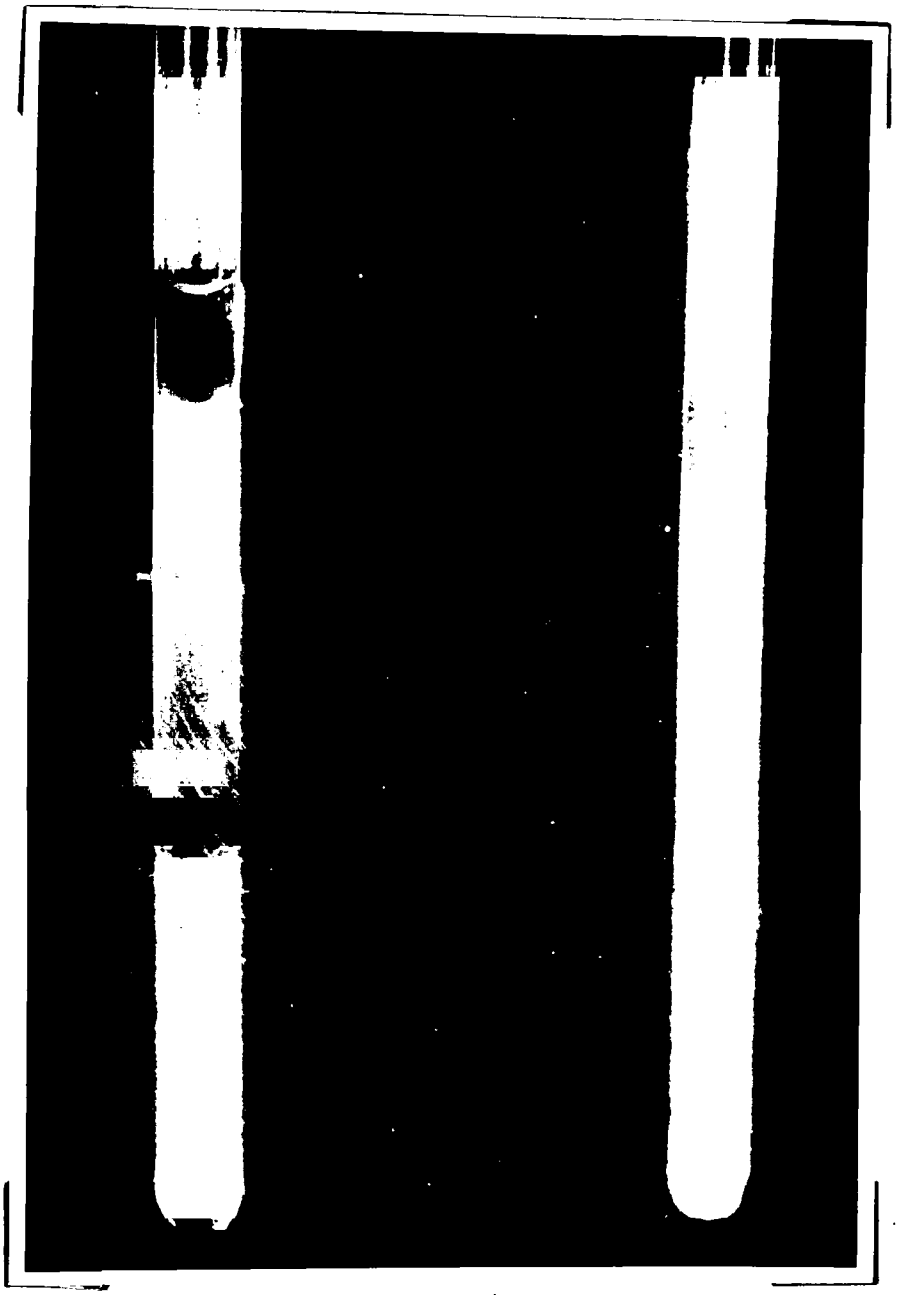


Plate 2. Agar double diffusion test showing three bands in positive tube and the other tube indicates negative normal serum control.

In another study Kagan and Bargai (1956) utilizing Melcher's antigen reported precipitin test to be positive after 20 days of infection of Trichinella and persisted for 65 days. Using saline extracts and alcoholic extracts of Ascaris positive ring test were reported by Gaur and Deo (1972) in ascariasis of pigs.

The number of components present in a given antigenic mixture can be resolved experimentally with various agar-serum techniques. Melcher's antigen when tested against sera of infected rats showed a minimum of 3 antigen components. Kagan and Bargai (1956) reported three antigenic components in trichinosis of rabbits whereas two antigenic components have been reported by Gaur and Deo (1972) in ascariasis of pigs.

IV. IMMUNE STATUS OF THE HOST

INTRODUCTION

One of the best methods of demonstration of immunity is the ability of the host to resist reinfection with the homologous parasite. This may either be complete which is rare in helminthic infections, or partial. Immune status of the previously exposed or infected host can be demonstrated by the effect it has on the parasites subsequent residence in that animal. Such effects of immunity on the nematode parasites have been reported as death, inhibition of growth and maturity, decrease in the egg and juvenile production, elimination of worms and reduced pathological conditions. The present study has been undertaken to ascertain the effects of immunization on the adult *S. gerri* worms during reinfection phase.

EXPERIMENTAL PROCEDURE

Three groups of rats marked A, B and C each consisting of 15 rats were selected in the present study. Groups A and B comprised those rats which were previously exposed to 3 female and 2 male adult worms and then challenged with 4 female and

3 male worms separately after 90 days and 65 days after primary infection. Group C consisted of rats infected with 4 female and 3 male adult worms and served as control. In all three groups regular microfilarial count was made, and rats were autopsied (5 rats/group) at intervals of 7, 14 and 28 days after infection.

RESULTS

It has been observed that during primary infection the microfilariae appeared in the peripheral blood circulation with a latent period of 7 ± 2 days and persisted for 65 ± 5 days in group A and B rats. The maximum microfilarial density in primary infection was recorded between 3rd and 4th weeks, thereafter it started declining. During secondary infection an appreciable and drastic reduction in level of microfilaraemia was recorded in both groups A and B. In both the groups the mean microfilarial density was comparatively much lower than that of primary infection; and the maximum microfilarial densities in both groups during primary and secondary infections were 5.6, 6.0 and 11.7, 13.3 respectively. However, normal course of microfilaraemia as observed in primary infection was recorded in control group C (Table I).

Table I. The effect of immunization on adult worms in secondary infection.

Weeks after infection	Recipient rats							
	Group A				Group B			
	Microfilariae/mm^3 of blood		Recovery of adult worms in sec. infection (%)		Microfilariae/mm^3 of blood		Recovery of adult worms in sec. infection (%)	
	Primary infection	Secondary infection	Live	Dead	Primary infection	Secondary infection	Live	Dead
1	0.3	1.4	100	-	0.2	0.6	33.3	66.6
2	5.1	3.2	25	75	4.6	2.0	16.6	83.6
3	10.2	5.6	-	--	11.0	4.6	-	-
4	11.7	5.0	-	100	13.3	6.0	-	100
5	10.0				8.1			
6	7.1				7.5			
7	5.5				9.1			
8	3.6				5.7			
9	4.1				0.5			
[illegible]	[illegible]				0.0			

Autopsy carried out 7 days after reinfection in group A and B showed 100% and 33.3% recovery of live adult worms respectively as compared to 100% of control group. One live female worm was found to have migrated to the vicinity of heart in one of the rats from group B. Rest of the worms were found either dead, exhausted or embedded in the peritoneal wall. Autopsy done after 14 days revealed maximum damage done to the adult worms in both the groups, and recovery of live adult worms in group A and B was dropped to 25.0% and 16.6% as compared to 75% recovery in the control group. In both the reinfected groups the worms were either dead or embedded in the mesentries, genital region or peritoneal wall forming worm knot (Plate 1). In group B, 50% of the worms were untraceable whereas only 25% in group A. The autopsy done after a month of infection showed no trace of adult worms, dead or live, in the peritoneal cavity of rats in both groups. Contrary to this 40% live worms were recovered in the peritoneal cavity in control group. About 20% of the worms were found exhausted or embedded in the peritoneal wall and genital region, and the rest were untraceable.

DISCUSSION

Although the resistant animal may not prevent the entry of the parasite into the body, the immune status of the host can be detected by the effect it has on the parasites subsequent

Plate 1. Autopsied rat showing dead and embedded worms in the peritoneal wall forming worm-knot in secondary infection.

residence in that animal. It is an established fact that a parasitic infection, unlike bacterial and viral infections, does not render the host completely immune with the homologous parasite, and only partial immunity develops which is manifested by the direct lethal effect on adult worms or its immature stages on subsequent exposure. Evidences are available (Sadun, 1947; Deo and Srivastava, 1961) which indicate that chickens once exposed to Ascaridia galli eggs become refractory to challenge reinfection after a month, and are able to eliminate all the worms of the test dose. Such immunity in chickens developed against primary infection lasted for at least 4 weeks to 2 months. There are many instances which indicate that primary exposure of animals to filarial worms render the host immune to challenge infection. Scott and Macdonald,(1951), Macdonald and Scott (1953, 1957) in a series of experiments showed that cotton rats acquired immunity against Litomosoides carinii following primary infection, and the immune status of the host persisted for one or two years after the introduction of primary infection. The principal effects of immunity to Litomosoides shown by the immune rats were (1) a reduction in number of worms due to growth failure of larval stages or to the death of developing worms, and (2) growth rate retardation.

In another study Ah et al. (1972) reported that the mongrel dogs previously exposed to Microfilaria larvae became

refractory to challenge 50 infective larvae 60 days after previous immunization. This was manifested by the delay in appearance of circulating microfilariae in vaccinated group as compared to control group; and the number of microfilariae were 1/10th of those of control. Similarly, Wong (1964) has reported that the dogs previously exposed to microfilariae of Mirofilaria induces the formation of antibodies which help in the destruction of microfilariae on subsequent administration.

The present study has also shown a marked reduction in microfilarial density and early disintegration of adult worms in resistant animals as compared to non-resistant control group. It is also of interest that rapid destruction and disintegration of adult worms in the peritoneal cavity starts earlier in group B rats as compared to group A. This is indicative of the fact that immunological activities persist in active form which are immediately triggered off by the secondary contact of the parasite. As a result, of local tissue reaction, the activities of the adult worms are restricted. Embedding of the worms in the mesentries, peritoneal wall, genital region finally leads to the death and disintegration of the worms. Similar results have been reported by Scott and Macdonald (1958) who observed that the growth of challenge infection of Litomosoides was retarded in those rats which still harboured some live worms; but little or no retardation of growth occurred in those where

only dead worms were found at autopsy.

Bertram (1966) reported that in case of reinfections, an immune response by the host imposed a retardation in the growth and development of new populations of Litomosoides worms particularly in the pleural cavity. This may also diminish the proportion of infective larvae which succeed in reaching the coelomic cavity.

In an exceptional case one live adult female worm was found to have migrated to the vicinity of the heart puncturing the diaphragm in secondary infection. This could be attributed to the unsuitable environment rendered by the primary infection that evoked the movement and migration of the parasite to other site. Bertram (1966) has reported that primary infection probably evokes an environment in the host unsuitable to worms by virtue of which Litomosoides worms, on reinfection, have been found to have migrated or moved away slightly from the normal site in the pleural cavity of the host.

Earlier reports indicate that the resulting phenomena - stunting of growth, failure of development to maturity, inhibition of reproduction, death and disintegration of worms - are due to interference with the nutrition of the parasite. Culbertson (1941) suggested that this might be designated the antienzyme basis of immunity to helminths. He further reported that the various antibodies particularly precipitin formed by the host

were instrumental in affecting the parasite. However, more recently it has been pointed out by Larsh and Race (1954), Cocker (1956), Olson (1959) and Taffs (1965) that antibodies which are known to be concerned with the prevention of feeding, stunting, immobilization, occasional death, expulsion of worms from host body, are wholly responsible, but there are other factors also known as cellular response playing an important role. Taffs (1965) has reported that cellular factors such as eosinophils and macrophages trap the activities of migrating Ascaris larvae in resistant host. Recently Bagai and Subrahmanyam (1970) have reported that the factors responsible for immunity in albino rats against Litomosoides carinii were not antibodies produced but the inflammatory cells (cell - mediated immunity) played a functional role in immunity. The evidence so far indicates that mechanism of immunity rests primarily on humoral factors with secondary cellular cooperation.

EXPERIMENTAL PROCEDURE

The drugs used as test chemicals in the present study are diethylcarbamazine citrate (hetrazan), tetramisole, thiabendazole, piperazine citrate, tetrachloroethylene and acetylarsan. Most of the drugs were administered orally except thiabendazole and acetylarsan which were inoculated intraperitoneally and intramuscularly respectively. The screening was initiated after three days of appearance of microfilariae, and continued till the microfilariae disappeared from the peripheral blood circulation. Complete disappearance of microfilariae from the peripheral blood circulation for three consecutive days was taken as an evidence of antifilarial action. Prior to the administration of drugs, weight of each rat was recorded.

After a week of the discontinuation of the drugs, the blood of rats was checked again to find any possibility of reappearance of microfilariae. At the close of the experiment, the rats were autopsied. A batch of ten non-medicated rats served as control was also kept in the laboratory, and a record on microfilaraemia was maintained.

RESULTS

Table I shows the various drugs tested at different dosages and its effect on microfilariae. Microfilariae appeared in the peripheral blood circulation of all rats with a latent period of 7 ± 2 days and continued to exist for 65 ± 5 days in control rats. An average density of 99 microfilariae/mm^3 was recorded in these rats.

The use of diethylcarbamazine citrate (DEC) in experimental rats brought speedy disappearance of microfilariae from peripheral blood circulation. All rats cleared of microfilariae from peripheral blood circulation in 3-6 days at the dose of 100 mg/kg body weight given twice daily presenting a 100% response. The onset of antifilarial action, however, was delayed by 7-9 days and 10 - 13 days at the dosages of 50 mg/kg and 25 mg/kg with 90% and 50% response in respective cases (Table I). No side effect of any kind was evident at these dosages.

Tetramisole, a broad spectrum anthelmintic, was tested for the first time in the present experiment. The drug showed potent microfilaricidal property. The drug when administered orally in 7.5 mg/kg and 15.0 mg/kg body weight (single dose/day) produced complete elimination of microfilariae from peripheral blood circulation of all rats (response 100%). Disappearance

Table I. Effect of various chemotherapeutic agents against microfilariae and adult worms of Setar

Drugs	Dose mg/kg body weight	No. of daily doses/day	Route	Time of disappearance of microfilariae (days)	Duration of medication (days)	Rats cleared of microfilariae
Untreated control	-	-	-	-	-	0/10
Diethylcarbamazine citrate	25.0	2	Oral	10 - 13	12 - 15	5/10
	50.0	2		7 - 9	9 - 11	9/10
	100.0	2		3 - 6	5 - 8	10/10
Tetramisole	7.5	1	Oral	3 - 5	5 - 7	10/10
	15.0	1		2 - 3	4 - 5	10/10
Thiabendazole	25.0	2	Oral/ Intraperitoneal	Nil	20	0/10
	50.0	2		Nil	20	0/10
	100.0	2		Nil	20	0/10
Acetylarsan	0.8	1	Intramuscular	Nil	20	0/10
	5.0	1		Nil	1	0/10
	25.0	1		Nil	1	0/10
Tetrachloroethylene	150.0	1	Oral	Nil	20	0/10
	300.0	1		Nil	1	0/10
	500.0	1		Nil	1	0/10
Piperazine citrate	100.0	1	Oral	Nil	20	0/10
	200.0	1		Nil	20	0/10

time of microfilariae at 15.0 mg/kg varied from 2-3 days while slightly delayed by 3-5 days at the dose of 7.5 mg/kg (Table I). The chemical also showed no side effect of any kind.

The drugs mentioned not only brought microfilarial elimination but the level of microfilaraemia also reduced considerably. Maximum microfilarial density in hetrazan and tetramisole treated cases, on an average varied between 3.8 and 4.5, and microfilarial population showed a declining tendency (Fig. 1). Microfilariae reappeared after a lapse of one week of discontinuation of drug in all hetrazan treated rats whereas no microfilariae were traced in rats treated with tetramisole.

Thiabendazole, another broad spectrum anthelmintic when tested in the present experiment at various dosages given orally and intraperitoneally proved completely ineffective even at higher therapeutic doses (Table I). The microfilarial population continued to increase even at higher doses and followed the microfilarial curve pattern of control rats (Fig. 1). Piperazine citrate has been noted to be completely ineffective on microfilariae as well as adult worms. Microfilariae never disappeared and microfilaraemia continued to follow the normal pattern (Fig. 2).

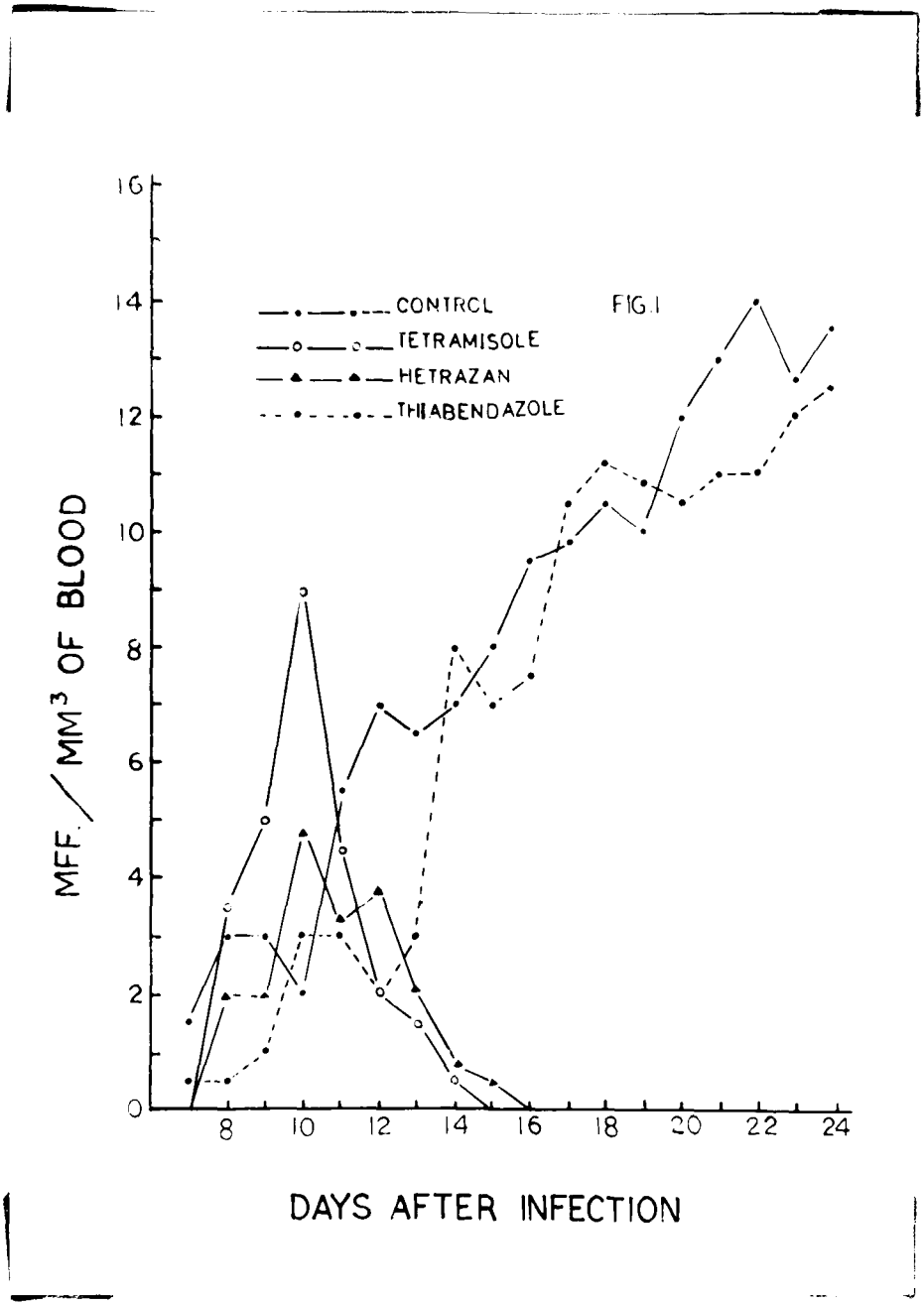


Fig. 1. The effect of anthelmintics on the microfilarial population of *S. cervi* in the blood of white rats.

Tetrachloroethylene on higher dosages such as 300 and 500 mg/kg body weight proved lethal and instant death of rats occurred. Lower dose (150 mg/kg) did not show any effect on microfilariae (Table I; Fig. 2). Another drug acetylarsan also showed ineffectiveness in this infection. Higher doses of the drug such as 25 mg/kg and 5 mg/kg body weight showed toxic effects on rats and the treated rats died over a period of 7-12 hours of administration. Hence these dosages were discontinued. Lower dose of 0.8 mg/kg did not show any effect on microfilariae even after 20 days of continued medication (Table I; Fig. 2).

The rats were autopsied after the treatment was over. Metrazan treated groups of rats were sacrificed over a period of 22-30 days from the initial infection. Average recovery of worms in these rats was 36%, 40% and 48% at the dosages of 25 mg/kg, 50 mg/kg and 100 mg/kg respectively. The remaining worms were either dead, exhausted or untraceable. Tetramisole treated groups were taken up earlier between 22-24 days because of its extreme efficacy. Both of its doses used produced sufficient mortality, and only 32% and 28% live worms were recovered (Table I). In case of thiabendazole and piperazine citrate treated groups live worms recovery, on an average, was 48-52% and 40-50% respectively. In case of acetylarsan and tetrachloroethylene autopsy was done between 1-20 days after

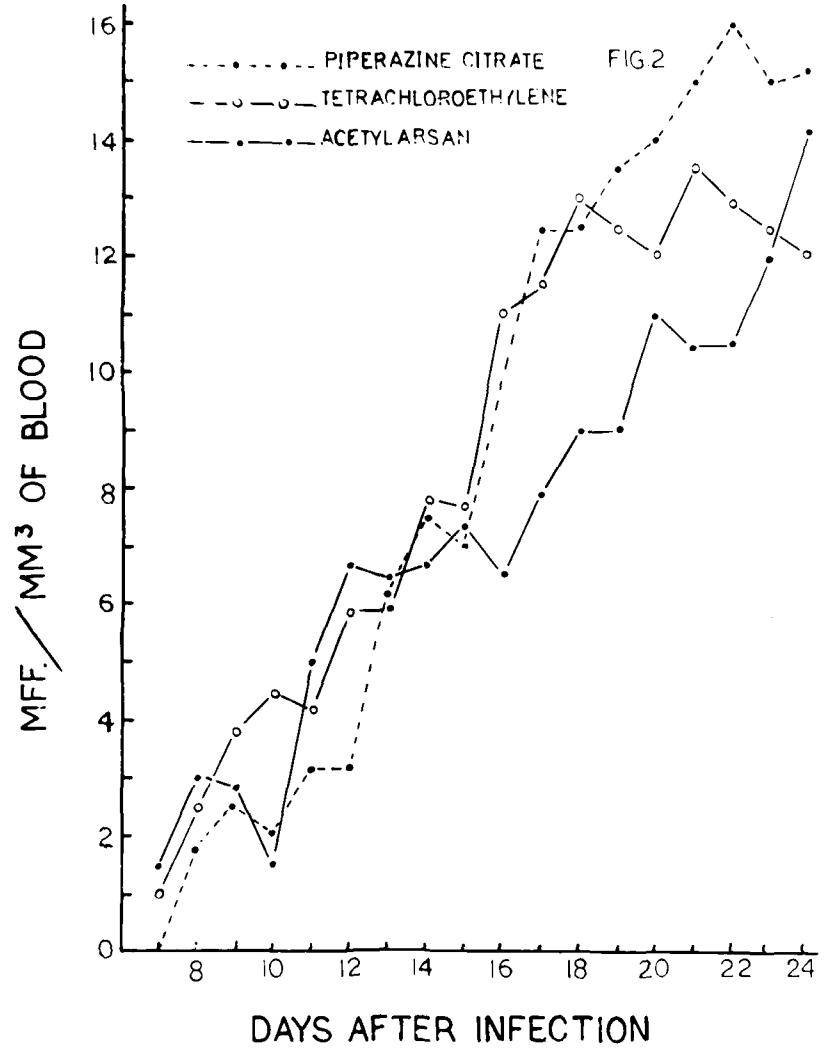


Fig. 2. The effect of anthelmintics on the microfilarial population of S. cervi in the blood of white rats.

treatment and recoveries of live adult worms in these cases were 52-100% and 42-100%. Average recovery of live adult worms in untreated control group had been 40% after 30 days of initial infection (Table I).

DISCUSSION

Diethylcarbamazine citrate (DEC) has been a drug of choice in most of the animal and human filariases. Burch and Ashburn (1951) reported the filaricidal action of hetrazan on microfilariae of Onchocerca volvulus but remained ineffective on adult worms. Cherry (1960), Yan et al. (1975) reported hetrazan as a superior and most effective drug in onchocerciasis and wuchereriasis. The allergic reactions, they reported, either could be successfully controlled by prednisolone or use of diethylcarbamazine-medicated salt. The drug has been found to be effective on microfilariae of Litomosoides and Brugia pahangi in experimental infections (Lammler et al., 1971; Zaman and Natarajan, 1973). Taylor and Terry (1960) reported that the drug was ineffective completely on embryonic stages and adult worms of Litomosoides carinii.

Singhal et al. (1972, 1973) tested DEC against Setaria cervi in in vivo and in vitro, and found it quite effective against the microfilariae. They also observed a decrease in

the reproductive potential of the adult worms and attributed it to be the beneficial effect of the drug. The authors further observed that the drug was capable of producing 100% mortality of adult worms in *in vitro* at a dose of 750 µg/ml concentration. The present study supports the above observations in part. The drug has shown lethal effect on microfilariae, but remained quite ineffective on the adult worms in *in vivo* condition even at higher doses. The views expressed by Singhal *et al.* (1973) regarding the decrease in the reproductive potential as indicated by the absence of microfilariae from the blood circulation even in the presence of live adult worms, is based purely on imagination. Hawking *et al.* (1950) have already shown that microfilariae of *Litomosoides carinii* in the presence of hetrazan move away from the blood circulation and get collected in the liver, which are later destroyed by opsonin like action. Reappearance of microfilariae, they reported, in peripheral blood circulation could be due to migration of fresh microfilariae from pleural cavity.

Elaborate studies on the mode of action of hetrazan have been carried out in *Litomosoides* infection by Taylor (1960) and Schardein *et al.* (1966). Taylor (1960) has observed that hetrazan stick to the walls of the capillaries of the liver by their tails with few leucocytes adhering to their tails. The

microfilariae, she reported, are probably eliminated by way of phagocytosis from the peripheral blood circulation. The best possible explanation has been put forward recently by Schardein et al. (1968). They have reported that within 20 minutes of the DEC treatment the blood circulating microfilariae get collected in the sinusoids or localized within the hepatic cells. The drug exerts its effect on microfilariae through a process of lysis, perhaps through loss of the sheath; and within 4 hours the extracellular forms are phagocytized by by Kupffer cells and neutrophils. The intracellular forms appear to be normal except for the loss of sheath and apparently escape phagocytosis which possibly be considered as a source of infection and reappearance.

Similarly, Laxmler et al. (1971) and Zaman and Natarajan (1973) have reported that hetrazan is quite effective on microfilariae of Litomosoides and Brugia malayi and adult worms remain unaffected. The characteristic reappearance of microfilariae in the peripheral blood circulation after a week of discontinuation of the drug in the present study is indicative of the fact that adult worms have never lost the reproductive potential but continue to produce it throughout; and as the influence of drug is over, the microfilariae reappear in the peripheral blood circulation after a brief lapse. Although DEC appears to be quite effective against microfilariae, but there

exists a dose-dependent relationship. With the decrease of the dosage, the response also decreases proportionately.

The other test chemical, tetramisole, used in this experiment, has been in extensive use as a broad spectrum anthelmintic against a wide variety of nematode infections (Thienpont et al., 1966; Bosseche and Janssen, 1967, Campbell and Hartman, 1968; Lammler et al., 1971). The drug showed pronounced and miraculous efficacy on the microfilariae of Setaria servi also. The microfilariae unlike hetrazan cases, disappear completely from peripheral blood circulation at all doses of tetramisole administered. Also, the onset of microfilaricidal action has been recorded much earlier than diethylcarbamazine. All rats treated clear of microfilariae presenting a 100% response at all dosages, while in DEC treated groups the response at various dosages range from 50-100%. It is also noteworthy that even lower dose of tetramisole (7.5 mg/kg) is as effective as the higher dose (100 mg/kg) of DEC, and in both cases response is 100%. Although DEC and tetramisole are both effective against the microfilariae but the latter has definite advantage because of its effectiveness even at lower doses. Further the drug has also shown some lethal effect on adult worms by virtue of which the percentage of live worms recovered on autopsy was only 28-32%, a condition comparable to untreated control group. Further, non-reappearance of

microfilariae after a week of discontinuation of drug reveals the persistence of drug's influence in the host body. These observations are undoubtedly in accordance with earlier investigation made by Lammler et al. (1971). The authors made a comparative screening of a number of anthelmintics in Meriones patelensis infected with Litomosoides carinii, and reported that DEC was effective on microfilariae only whereas tetramisole was highly effective against microfilariae as well as adult worms.

The drug has been reported to be tolerable even at several times recommended dose of 2.5 mg/kg (Thienpont et al., 1969). Ozcan (1967) reported that higher dose of tetramisole such as 20, 30 mg/kg body weight produced certain transient side effects in experimental trichinosis in mice. In vitro study made by Thienpont et al. (1966) reveals that the drug exerts a rapid paralysing action on various species of nematode.

Thiabendazole, another broad spectrum anthelmintic (Leiper and Crowley, 1963; Yakstis et al., 1968; Campbell and Hartmann, 1968; Macdougall, 1969) when tested on the experimental rats proved completely ineffective. None of the rats cleared of microfilariae even for 20 days of continued medication. Further, the recovery of adult worms on autopsy was also 48-52% which were a fairly high figures indicating the inefficacy of

the drug in such infection. However, Singhal *et al.* (1973) have observed positive action of thiabendazole against adult *Setaria cervi* in *in vitro* study. The ineffectiveness of thiabendazole as observed in the present experiment is undoubtedly in accordance with the earlier report by Knochiri (1966) in patients with *Loa*, *Acanthocheilonema* and *Onchocerca* infections. He tested thiabendazole on patients in a dose of 25 mg/kg for 5–10 days and found it to be ineffective. Larger doses increased the frequency of toxic effects in patients.

The other drugs such as tetrachloroethylene, piperazine citrate and acetylarsan which have been reported to be highly effective against *Ancylostoma*, *Nippostrongylus*, *Oesophagostomum*, *Ascaridia*, *Heterakis* infections (Steward, 1955; Chumard and Eveleth, 1955; Alicata, 1958; Jeffery *et al.*, 1962; Morley and Thompson, 1963; Rana *et al.*, 1965) proved completely ineffective in *Setaria cervi* infection. These observations are in accordance with the earlier investigators (Singhal *et al.*, 1972) who showed complete inefficacy of these drugs in rat-cervi system. However, they (Singhal *et al.*, 1973) have reported that acetylarsan is effective on adult worms in *in vitro* condition. In the present *in vivo* study these drugs have been found to be completely ineffective on microfilariae and adult worms even at higher therapeutic doses. Higher doses in certain cases showed increased toxic effects and mortality with no antifilaricidal action.

In vivo screening of these drugs using *Setaria cervi* as test organism has been very satisfactory. Diethylcarbamazine and tetramisole proved to be chemicals of considerable importance. Both the chemicals have shown the capability of destruction of circulating microfilariae in the blood of experimental host. These could, therefore, be recommended as useful drugs against natural *Setaria cervi* infection; and a breakthrough could be achieved by reducing the risk of transmission. Since tetramisole has shown its effectiveness against the adult worms also, this may be taken as the drug of choice.

SUMMARY

The filariid worm, Setaria cervi, was experimentally implanted in laboratory white rats with test doses ranging from 2-12 worms per rat. Microfilariae appeared in the peripheral blood circulation of all rats with a latent period of 7 ± 2 days and microfilaraemia persisted for 65 ± 5 days. Live worms were recovered from the peritoneal cavity upto 4-6 weeks after initial infection.

White rats thus infected manifested positive changes in blood. Leucocytes such as eosinophils, neutrophils and lymphocytes were found to have increased. The highest microfilarial density generally coincided with the greatest eosinophilic response. The qualitative and quantitative serum protein analyses of infected rats showed appreciable increase in total globulin and its fractions, a net decrease in albumin and an overall increase in total serum protein. α_1 and γ - globulins showed appreciable increase and non-specifically incriminated for the production of antibodies.

Infected rats have shown ability to produce various kinds of antibodies detectable by known immunologic procedures. White rats showed capability of producing anaphylactic or homocytotropic antibody detectable by passive cutaneous

anaphylactic procedure using short sensitization period. Anaphylactic antibody was detected from 2-14 weeks from initial infection. Rat immune sera failed to sensitise rabbit skin indicating absence of heterologous PCA reactions.

Complement fixing antibodies against Setaria servi infection was detected through CFT during 2nd week and continued for 7 weeks with a peak titer of 1:80 during the 4th week. During secondary infection with a slightly higher challenging test dose, higher antibody titer was noted as compared to primary infection. The rise and fall of antibody titer has been found to coincide with the maximum microfilarial concentration (3rd and 4th weeks) and also with the survival of adult worms in the peritoneal cavity of the rats.

With the utilization of precipitin tests precipitin antibodies have also been detected in the immune serum. The antigen titer obtained through precipitin ring test was detected from 1-6 weeks of initial infection with maximum titers during 3-5 weeks. Preer's double diffusion technique in one dimension showed that soluble S. servi antigen (Melcher's antigen) contained at least three antigenic components as three precipitin bands were observed against immune serum.

The rats infected with S. servi developed some sort of immunity which was characterised by the lethal effects shown by

the host on subsequent administration of the parasite. Because of immune effects the level of microfilaraemia and survival period of adult worms considerably reduced in secondary infection. Adult worms were found embedded in the peritoneal wall, mesentries and genital region as a result of some sort of tissue reaction.

Different drugs namely hetrazan, tetramisole, piperazine citrate, tetrachloroethylene, acetylarsan and thiabendazole were tested in rat-cervi system. Tetramisole and hetrazan have been recorded to be highly effective in this infection. Both the drugs showed the microfilaricidal action without apparent side effects. Tetramisole, for the first time tested in this infection, showed comparatively high chemotherapeutic value by showing its efficacy on the adult worms also. Other test chemicals have been found to be ineffective or too toxic.

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